THE EVOLUTION OF COLOR POLYMORPHISMS IN COLIAS BUTTERFLIES: PREFERENCES, LEARNING, AND SENSORY LIMITATIONS

by

Lisa Limeri

B.S. in Ecology, Evolution, and Behavior, Binghamton University, 2012

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This dissertation was presented

by

Lisa Limeri

It was defended on

April 20, 2017

and approved by

Nathan I. Morehouse, PhD, Assistant Professor

Walter P. Carson, PhD, Associate Professor

Corinne Richards-Zawacki, PhD, Associate Professor

Nathan Clark, PhD, Assistant Professor

Thomas N. Sherratt, PhD

Dissertation Advisor: Nathan I. Morehouse, PhD, Assistant Professor

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Lisa Limeri, PhD

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Polymorphisms are useful for studying major evolutionary questions, such as how diversity arises and is maintained over time. They are a widespread form of biodiversity and take on a number of different forms, including behavioral, physiological and morphological. The female-limited ‘alba’ color polymorphism in the butterfly family Coliadinae is a widespread polymorphism, yet despite its prevalence, the selective forces that maintain it are not fully understood. In order to better understand the evolutionary mechanisms that maintain this ubiquitous color polymorphism, I first utilized a phylogenetic approach to answer questions about the evolutionary origin and history of the ‘alba’ polymorphism. Ancestral state reconstruction revealed that the ‘alba’ polymorphism is ancestral to the Coliadinae, but is often lost, leaving about half of the current species monomorphic. Next, I asked whether sensory limitations might contribute to polymorphism maintenance via the influence of reproductive interference on male mate preferences. To answer this question, I modeled the male visual system to determine whether males should experience difficulty discriminating between the white female morph (‘alba’ morph) and other co-flying white butterflies. I found that visual limitations may explain a male mate preference for the more discriminable, yellow (non-‘alba’) female morph. I then tested whether such a preference exists and whether males modify their mate preference based on the frequency of each morph in the population. Field observations of natural populations and behavioral experiments in

captive populations revealed that males do prefer to court non-‘alba’ females, and that this preference is unaffected by morph frequency. Finally, I used a theoretical model that combined signal detection theory and optimal diet theory to explore optimal decision making in situations where options differ in value and discriminability. This model revealed that the morph ratio, discriminability, and recognition costs should influence optimal mate preference and that mate preferences can affect polymorphism maintenance by affecting morph fitness. Altogether, these studies advance our understanding of the role that the evolutionary history, sensory limitations, and community composition have played in a widespread but poorly understood polymorphism.

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1.0 INTRODUCTION

How diversity arises and what maintains it over time are major questions in evolutionary biology. Polymorphisms are a common form of within-species diversity that have proven to be useful for understanding how evolutionary mechanisms maintain diversity. Color polymorphisms in particular are a longstanding model system for understanding how diversity is maintained or lost over time. Color polymorphisms lend themselves to such studies because they are easily observed and quantified, and they are found in a wide range of taxa (Forsman *et al.* 2008; McKinnon & Pierotti 2010). Here I use a well-known color polymorphism, the female-limited 'alba' polymorphism of butterflies in the sub-family Coliadinae, to understand the role of complex environments, sensory limitations, and mate choice in maintaining this particular form of color diversity.

Many species in the butterfly sub-family Coliadinae are characterized by a female-limited color polymorphism where the wings of some females are white, termed the 'alba' morph, whereas the wings of non-'alba' females are yellow or orange depending on the species. This polymorphism is genetically-controlled and appears to be a single-locus polymorphism where 'alba' is dominant over non-'alba' (Gerould 1923). Approximately half of the 203 species in the Coliadinae are polymorphic and these species are geographically widespread, with representative species on every continent except Antarctica (Braby 2000; Grieshuber, Worthy & Lamas 2012; Limeri &

Morehouse 2016). Despite the ubiquity of the ‘alba’ polymorphism, the mechanisms that maintain it over time are not fully understood. Prior work on several species suggest that ‘alba’ females have some developmental advantages over non-‘alba’ females because ‘alba’ females spend less nitrogen on developing nitrogen-rich pterin pigmentation than non-‘alba’ females (Watt 1973). This difference in resource allocation may serve as the basis for a number of advantages that ‘alba’ females might experience. For example, studies comparing ‘alba’ and non-‘alba’ females in several *Colias* species found that ‘alba’ females develop more quickly during the pupal stage, have higher body fat, and have more mature eggs when they emerge as adults (Graham, Watt & Gall 1980; Nielsen & Watt 1998). While ‘alba’ females may enjoy a developmental advantage, several researchers have reported a male mating bias towards non-‘alba’ females (Graham *et al.* 1980; Watt 1995; Kemp & Macedonia 2006). Thus, ‘alba’ females may benefit by freeing up resources that would otherwise be devoted to wing pigmentation, but in the process become less attractive to mate-searching males. Researchers have proposed that this mating disadvantage for ‘alba’ females may be the result of reproductive interference between ‘alba’ females and heterospecific co-occurring white butterflies, but this hypothesis has never been formally evaluated (Nielsen & Watt 1998, 2000).

While previous work has provided important insights into this widespread polymorphism, there are a number of key issues that remain unresolved. For example, the evolutionary origins and history of the ‘alba’ polymorphism are unknown. Further, while mate preferences have been hypothesized to play a role in the maintenance of the ‘alba’ polymorphism, how mate preferences are formed and how they impact female

fitness has never been empirically tested. Here I deepen our understanding of the ‘alba’ polymorphism by addressing these key questions. First, I set out to discover when the polymorphism arose and how often each morph is maintained or lost over time. Phylogenetics and comparative analyses are useful tools for elucidating selective pressures acting on a polymorphism. For example, Roulin and Wink (2004) provided strong evidence supporting hypothesis that predator–prey relationships played a role in the evolution of color polymorphism in raptors by examining the evolutionary history of raptors. These kinds of phylogenetic inferences provide essential clues into the origins and stability of polymorphisms. Therefore, I used a phylogenetic approach to test two hypotheses: (1) the ‘alba’ polymorphism is evolutionarily ancient and (2) the ‘alba’ polymorphism experiences many independent reversions to monomorphism. Our study joins others that have established similar phylogenetic patterns for color polymorphisms in systems such as raptors and poison dart frogs (Roulin & Wink 2004; Wang & Shaffer 2008; Penney *et al.* 2012).

I then empirically tested two mechanisms that have been hypothesized to maintain the ‘alba’ polymorphism. First, researchers have hypothesized that limitations of male visual discrimination abilities play a role because one of the morphs is thought to resemble other butterfly species in the community (Nielsen & Watt 2000). I evaluated this hypothesis by measuring colors of butterfly species that co-exist throughout North America. I then built a model of the *Colias* male visual system and used it to determine whether males should be able to easily discriminate between co-flying conspecific ‘alba’ and heterospecific white butterflies.

The second hypothesis I tested is that male mate preferences play a role in maintaining the ‘alba’ polymorphism. Multiple researcher have observed a male mating bias for non-‘alba’ females and speculate that this bias results from difficulty discriminating between conspecific ‘alba’ and heterospecific white butterflies (Graham *et al.* 1980; Nielsen & Watt 2000). There are two ways that this species could evolve to respond to difficulty discriminating ‘alba’ females from other white butterflies. One possibility would be for males to evolve a fixed preference for non-‘alba’ females so that males always prefer to mate with the more discriminable option. Alternatively, males could evolve to have flexible preferences that adjust based on males learning the morph frequency in their community. A final possibility is that males may evolve to have preferences that are a combination of both of these mechanisms, where males possess an innate bias, but can modify it based on prior experience as they learn about the community composition. Which evolutionary circumstances favor learning, innate biases, or a combined strategy is long-standing question in evolutionary biology (Dukas 2006; Verzijden *et al.* 2012). In the Coliadinae, whether male preferences are flexible and affected by morph frequency has never been formally evaluated. Therefore I tested whether males have a mating preference for ‘alba’ or non-‘alba’ females and whether this preference is affected by prior experience. I investigated this hypothesis using both correlational observations of extant field populations and behavioral experiments with lab-reared individuals.

Finally, using a theoretical approach, I explored optimal male mate preferences in response to female polymorphism under different environmental circumstances and how these preferences affect polymorphism stability. I modified and applied a

theoretical model (Getty 1985) to understand optimal mate preferences when some options are difficult to discriminate between. I expanded this model to explore which variables should affect male mate preferences and how male mate preferences impact female morph fitness over time. The resulting model is broadly applicable to decision-making scenarios where options differ in value and discriminability.

In the chapters that follow I describe this suite of approaches that I took to tackle these questions, including comparative analysis, mathematical modeling of the male visual system, behavioral assays, and theoretical modeling. Together, these studies helped increase our understanding of key questions about polymorphism maintenance over time. Some specific questions I addressed include how visual limitations may influence mate preferences, the role of fixed vs. learned mate preferences in maintaining diversity, and what factors influence optimal mate choice between polymorphic options.

2.0 THE EVOLUTIONARY HISTORY OF THE 'ALBA' POLYMORPHISM IN THE BUTTERFLY SUB-FAMILY COLIADINAE (LEPIDOPTERA: PIERIDAE)

2.1 INTRODUCTION

Polymorphisms, the presence of multiple distinct, genetically-determined phenotypes within a single interbreeding population, offer a number of key opportunities to study how selection shapes phenotypic diversity (Ford 1945). Because the fitness of morphs are often interdependent, emergent interactions between selection pressures and phenotypes arise that can deepen our understanding of evolution in complex systems (Gross 1996). For example, the fitness of each male mate strategy in the trimorphic side-blotched lizard (*Uta stansburiana*) depends on a number of selective pressures including neighbor identity and male hormone levels leading to regular temporal oscillations in relative morph frequency (Sinervo & Lively 1996; Mills *et al.* 2008). Polymorphisms provide unique insight into how interactions between selective pressures maintain diversity.

One strategy to understanding polymorphisms is to consider them in a comparative framework. This research perspective allows us to identify both ancestral states and patterns of evolutionary origins or losses. Knowledge of the evolutionary history of polymorphic traits has deepened our understanding of a wide variety of evolutionary processes. For example, researchers have proposed that polymorphisms promote speciation (McLean & Stuart-Fox 2014), and several phylogenetic studies have

supported this claim (Corl *et al.* 2010; Hugall & Stuart-Fox 2012). In side-blotched lizards, the repeated loss of one morph is associated with increased rates of phenotypic change and increased divergence between populations (Corl *et al.* 2010). Corl et al. (2010) suggest that this may play a role in speciation. Comparative studies of polymorphic traits have also illuminated the relationship between phenotypic diversity and niche breadth. Other research suggests that disruptive selection plays an important role in maintaining polymorphisms (Galeotti & Rubolini 2004). A large-scale investigation of owls, nightjars, and raptors supports this hypothesis by revealing that polymorphic species occupy a wider niche breadth than monomorphic species (Galeotti & Rubolini 2004).

We took a comparative approach to understanding the female-limited ‘alba’ color polymorphism in the butterfly sub-family Coliadinae. Females of many species may be various shades of yellow/orange/red, caused by different combinations of pterin pigments. Conversely, the ‘alba’ female morph (named after the Latin word for white) is paler in appearance due to reduced and/or altered expression of pterin pigmentation on wing surfaces (Watt, 1973; Fig. 1). Males of most species are shades of yellows, oranges, or reds, similar to the non-‘alba’ female morph, though they may differ in wing pattern and/or UV reflectance (Kemp, Rutowski & Mendoza 2005).

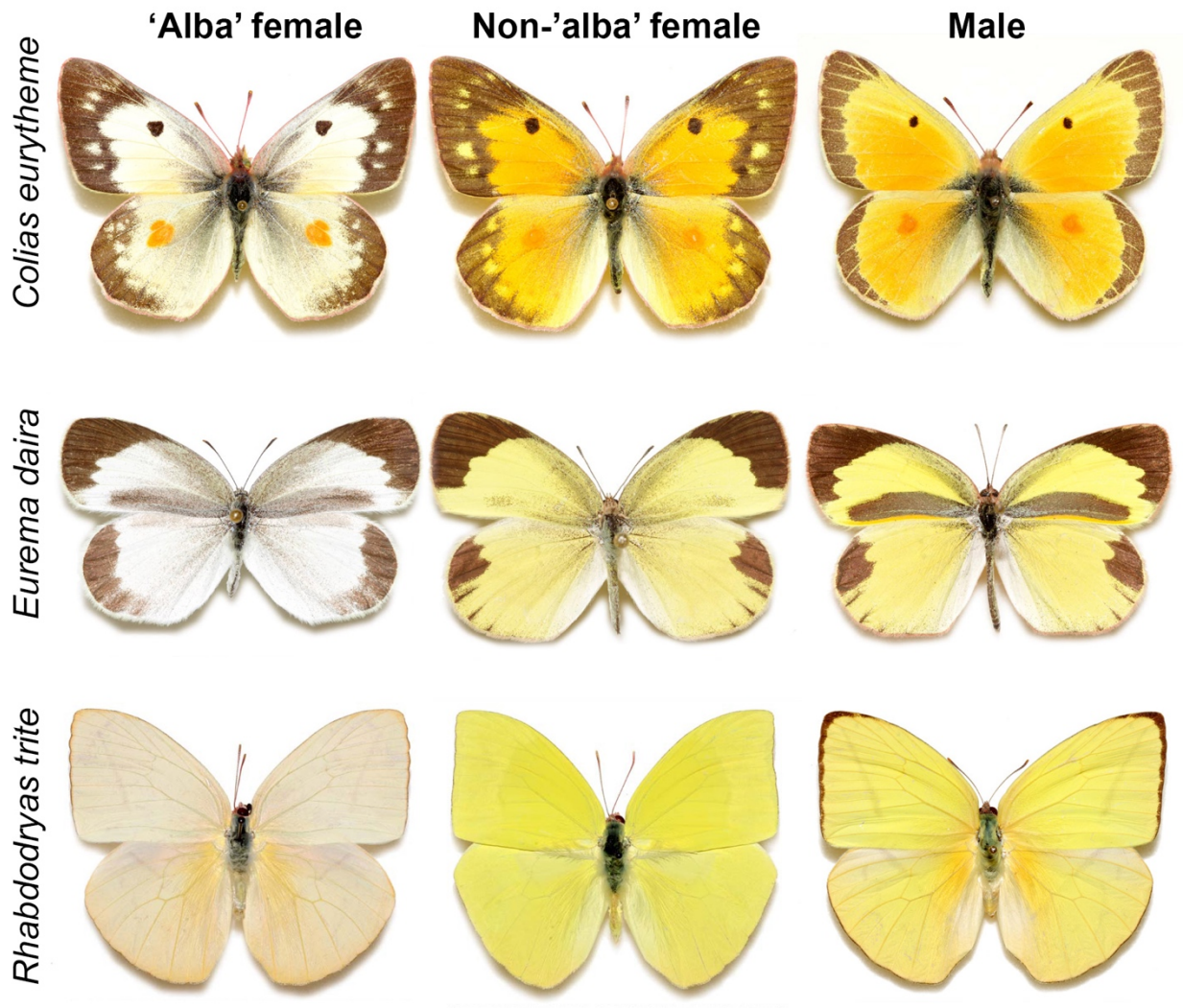

Figure 1. Examples of phenotypic forms of three species in the Coliadinae.

The 'alba' female phenotype (left column), the non-'alba' female phenotype (center column) and the male phenotype (right column) are pictured for three different species: *Colias eurytheme* (top row), *Eurema daira* (center row) and *Rhabdodryas trite* (bottom row). Photographs © Kim Davis, Mike Stangeland, & Andrew Warren.

Polymorphic species in this group are found worldwide (Braby 2000; Grieshuber *et al.* 2012). Despite the prevalence of this polymorphism, little is known about its

evolutionary history (Braby, Vila & Pierce 2006). In addition, the factors contributing to its maintenance within particular species and/or populations remain the subject of experimental inquiry and debate (Graham *et al.* 1980; Nielsen & Watt 1998; Kemp & Macedonia 2006; Limeri & Morehouse 2014). Polymorphic species have been reported from most of the 18 genera within the Coliadinae, though this data has never before been formally summarized. Questions of current selection on this polymorphism have come from studies of several species in the genus *Colias* (Graham *et al.* 1980; Nielsen & Watt 2000). For example, 'alba' females from the species *Colias eurytheme* benefit from a developmental advantage over non-'alba' females by redirecting 0.4mg of guanosine triphosphate (GTP) from pigment synthesis to other metabolic processes during pupal development (Watt 1973). This results in 'alba' females developing faster in cold climates, having higher fat body reserves at all ages and possessing more mature eggs at pupal emergence than non-'alba' females (Graham *et al.* 1980). Graham *et al.* (1980) have argued that the developmental advantage of 'alba' females is offset by reduced male interest in 'alba' females during mate searching. Subsequent work to test this hypothesis revealed that the selective pressures acting on 'alba' and non-'alba' morphs are strongly context-dependent (Graham *et al.* 1980; Watt, Kremen & Carter 1989; Nielsen & Watt 2000). Fitness advantages associated with the 'alba' morph may differ in strength and direction depending on microhabitat temperature and/or the community of interacting species (Watt *et al.* 1989; Nielsen & Watt 2000). For example, co-occurring species that visually resemble the 'alba' phenotype may act as interference competitors during early stages of mate searching (Graham *et al.,* 1980; Limeri & Morehouse, 2014), leading to stronger selection against the 'alba' phenotype in

communities with high densities of these interference competitors. The context-dependence of these selective pressures suggests that the ‘alba’ phenotype may have transient benefits or the benefits associated with each phenotype may fluctuate dramatically depending on environmental factors, which could lead to sudden fixation of one form or the other. How this environmental context-dependence has influenced the evolutionary trajectories of the ‘alba’ polymorphism across this clade is not well understood.

Despite numerous studies about the contemporary consequences of the ‘alba’ polymorphism, we do not know when or how often it arose. A comparative approach can reveal the origin(s) and evolutionary patterns of the ‘alba’ polymorphism. Since nearly all currently available information about the ‘alba’ polymorphism arises from studies of a few species within the *Colias* genus (Graham *et al.* 1980; Nielsen & Watt 2000; Kemp & Macedonia 2006), information about the evolutionary origin and history of the ‘alba’ polymorphism may be valuable at the level of the *Colias* genus as well as the entire sub-family.

Here, we use ancestral state reconstructions on three different phylogenies to discriminate between alternative hypotheses regarding the ancestral state of the Coliadinae: (1) the ancestral state was the ‘alba’ morph, (2) the non-‘alba’ morph, or (3) polymorphic for both morphs. In addition, we evaluated these three alternative hypotheses specifically for the genus *Colias*, the most species-rich genus in the Coliadinae. Furthermore, we evaluated the relative stability of the ‘alba’ polymorphism over time by looking at the number of gains and losses across the Coliadinae.

2.2 MATERIALS AND METHODS

2.2.1 Species list

A comprehensive list of the 18 genera and 203 species encompassed by the sub-family Coliadinae was compiled using primarily 3 sources: an exhaustive catalogue by Charles Bridges (1988), Catalogue of Life online database (taxonomic scrutiny of Coliadinae by Haeuser, Holstein and Steiner; Roskov *et al.*, 2015) and an online catalogue of butterflies in North and Central America (Warren *et al.* 2012). We considered a species name valid if it appeared in one of these three main sources and one additional source (see Supplemental Data). Exceptions to this rule and uncertain taxa are outlined below.

The genus *Colias* is particularly well-studied and the taxonomy of *Colias* was extracted directly and exclusively from a recent monograph by Grieshuber, Worthy, and Lamas (2012). Similarly, a recent revision of the genus *Gonepteryx* by Reinhardt (2002) presents a complete description of the genus, so we adopted this taxonomy in our study.

The taxonomic status of several species within the Coliadinae remains poorly established. Following Davis and Barnes (1991), we treated *Catopsilia grandidieri* [Mabille, 1877] as a synonym to *C. thauruma* [Reakirt, 1866]. We treat *Eurema regularis* [Butler, 1876] as a subspecies of *E. desjardinsii* [Boisduval, 1833] following Yata's revision of the genus *Eurema* (1989) and Larsen (2005). The genus *Teriocolias* [Röber, 1909] is also not well resolved and there is no recent review. Following the most recent

sources, we consider *T. zelia* a valid species name with *T. zelia riojana* and *T. zelia andina* as subspecies (Vargas 2012).

2.2.2 Data collection

For each species we recorded the phenotypes present in museum specimens, books, and online databases. We defined the ‘alba’ morph as the female variant in which the ground color on all wing surfaces is changed from male-like (yellow, orange, or red, depending on the species) to white, presumably due to a change in pterin pigmentation. Given that it was not possible to directly assess pterin pigmentation via the images used during data collection, ‘alba’ morphs identified in our study may be pale or white for a variety of reasons, including complete loss of pterin pigmentation, alteration of pterin profiles, or other modifications to the pigmentary basis of female coloration. The non-‘alba’ morph, then, was defined as any phenotype in which the ground color was not white on all wing surfaces. When a species contained some ‘alba’ females and some non-‘alba’ females, it was considered to be ‘alba’ polymorphic. When all females were the ‘alba’ morph, it was considered monomorphic for the ‘alba’ morph. When all females were characterized by yellow/orange/red pigments on some or all wing surfaces, the species was considered monomorphic for the non-‘alba’ phenotype.

This dataset is prone to a few biases. Morph presence data was collected based on pictures and reports from a variety of websites, collections, journal articles, and books (see Supplemental Data). Consequently, our dataset is vulnerable to species identification mistakes, especially for rare species and similarly-appearing species.

Furthermore, a morph was considered absent if there were no positive reports, which may lead to an underestimation of the prevalence of rare morphs and artificially inflate the proportion of species that were considered monomorphic. Together, these factors may result in an underestimation of the prevalence of polymorphism. We were unable to find information on 13 of the 203 species in the Coliadinae and these species were excluded from all analyses.

2.2.3 Ancestral state reconstruction

We performed ancestral state reconstructions on three different phylogenetic trees: a topology resolved to the genus level, a topology including all species, and a phylogeny produced using molecular data from a subset of species for which sequences were available. The genus-level topology and the topology including all species in the Coliadinae were derived from a molecular phylogeny published by Braby et al. (2006). Three of the 18 genera were not included in their molecular phylogeny (*Abaeis*, *Prestonia*, & *Rhabdodryas*) and their positions in the final topology are based on morphological characters as discussed by the authors (Braby *et al.* 2006). This placement yields a polytomy of these three genera. To ensure that this uncertainty did not influence results, we performed ancestral state reconstructions on all three possible resolutions as well as the polytomous arrangement. The arrangement of these genera did not affect the outcome of analyses, and results are shown with the polytomy.

All ancestral state reconstructions were performed in the program Mesquite V2.75 (Maddison & Maddison 2011) using maximum likelihood methods with a general Mk1 model. Each node is estimated independently with a decision threshold of t=2.0.

The goal of the genus-level analysis was to examine the distribution of polymorphic species on a large scale. Accordingly, genera were coded as “polymorphic” if any of the species in the genus exhibited the ‘alba’ polymorphism, and “monomorphic” if all of the species in the genus were monomorphic.

In the topology including all species (species-level analysis), each genus was analyzed as a polytomy of all its species because the species relationships within genera are not resolved. Each species was assigned a character state of “polymorphic,” “monomorphic ‘alba,’” or “monomorphic non-‘alba’” as described above.

The molecular phylogeny was produced using sequences from one mitochondrial gene (cytochrome c oxidase subunit I, *COI*) and one nuclear gene (elongation factor 1a, *ef1a*). Both these genes exhibit evolutionary rates suitable for examining differences at the genus level (Braby *et al.* 2006). The molecular phylogeny included all species in the Coliadinae for which the sequences for *COI* and *ef1a* were available: 47 sequences from 35 species encompassing 15 of 18 genera. The sequences were aligned, trimmed, and concatenated in the program SeaView V4.4.2 (Gouy, Guindon & Gascuel 2010). The resulting sequences consisted of 1128 characters, of which 838 were informative. The sequences were analyzed in Mr. Bayes (Ronquist & Huelsenbeck 2003) using Markov Chain Monte Carlo analysis. We ran two chains using a general time-reversible model with 10 million generations and 4 gamma categories. The majority rules

consensus tree was used for the ancestral trait reconstruction in Mesquite. Figures were produced using the program FigTree (Rambaut 2012).

2.3 RESULTS

This is the first formal summary of the pervasiveness of the ‘alba’ polymorphism throughout the Coliadinae. The Coliadinae contains 203 species dispersed throughout 18 genera, with relevant color morph character states available for 190 out of these 203. Seventy-two percent (13/18) of the genera contain polymorphic species. Approximately half of the species in the Coliadinae are polymorphic (88/190; 46%).

2.3.1 Topology resolved to genus level

Ancestral reconstruction of the topology resolved to the genus level indicates overwhelmingly that the ancestor of the Coliadinae was a polymorphic species (Fig. 2). Only five of the 18 genera (28%) contain no polymorphic species and they are phylogenetically dispersed. These results indicate that polymorphism is likely the ancestral state and that repeated reversions to monomorphism have occurred.

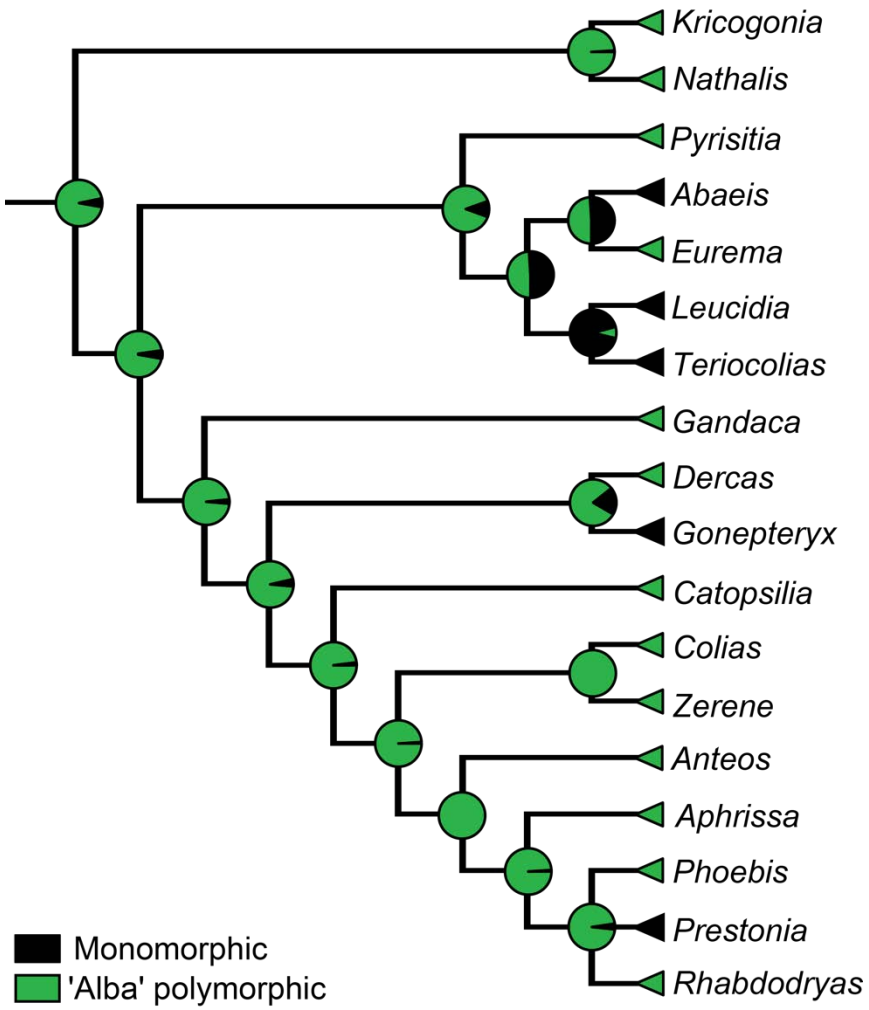

Figure 2. Genus-level ancestral state reconstruction

Genus-level ancestral state reconstruction performed on a topology of the 18 genera in the sub-family Coliadinae. Green wedges indicate that at least one polymorphic species is present in the genus whereas black wedges indicate that all species are fixed. The area of the slices of the pie charts at each internal node represent the probability of that ancestor's character state.

This analysis also reveals that the ancestor of the genus *Colias* is likely polymorphic. Species relationships within *Colias* are not resolved in this analysis, so it was not possible to draw conclusions about the polymorphism's stability within the genus *Colias*.

2.3.2 Topology resolved to species level

Ancestral reconstruction of the topology including all species reveals finer detail (Fig. 3). This analysis indicates equal likelihood that the ancestor of the Coliadinae was polymorphic or monomorphic non-‘alba’ (0.37 each) and a lower probability that the ancestor was monomorphic ‘alba’ (0.26). This analysis also shows numerous transitions between polymorphism and monomorphism, indicating evolutionary instability.

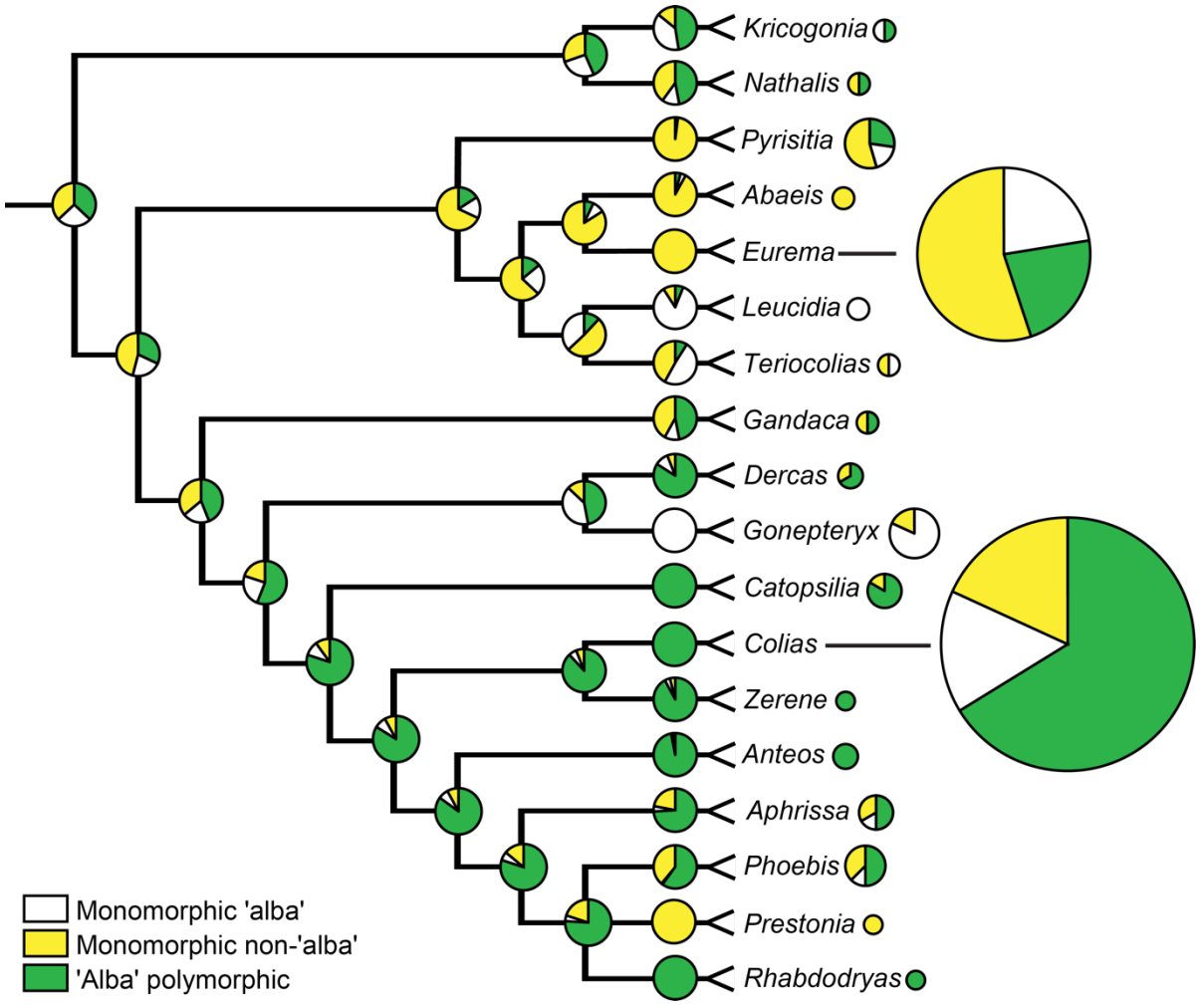

Figure 3. Species-level ancestral state reconstruction

Species-level ancestral state reconstruction performed on a topology including all 190 species in the Coliadinae treated as polytomies within each genus (polytomies not shown). Pie charts to the right of the genus names are scaled relative to the number of species within the genus, ranging from 1 (*Prestonia*) to 77 (*Colias*). The slices of the pie represent how many contemporary species in the genus represent each character state. The circles to the left of the genus names at internal nodes on the tree represent the relative probabilities of the character state for that ancestor.

This analysis reveals with high certainty that the ancestor of the genus *Colias* was polymorphic. The species relationships within the *Colias* are not well resolved. However, the high proportion of monomorphic species (44%) indicates that the polymorphism has likely been lost repeatedly and is unstable. Monomorphic species are relatively evenly split between the two morph types (53% non-'alba', 47% 'alba').

2.3.3 Molecular phylogeny

The ancestral reconstruction of the molecular phylogeny reveals that the ancestor of the Coliadinae was likely a polymorphic species (Fig. 4). The phylogeny also suggests multiple losses of polymorphism within this phylogeny. The topology of this tree is similar to the topology produced by Braby et al. (2006), but differs in its placement of the clade composed of the genera *Aphrissa, Phoebis*, *Prestonia*, and *Rhabdodryas*. Braby places these genera as the sister clade to the genus *Anteos* whereas our phylogeny places them as the sister clade to the genus *Gandaca* (Braby *et al.* 2006). Future reconstructions including more sequences would likely provide improved resolution of this topology. However, it is unlikely that this discrepancy

affected results because our analyses on both our molecular phylogeny and on the topology produced by Braby et al. (2006) revealed qualitatively similar results: that the ancestor is polymorphic and there are repeated losses of polymorphism.

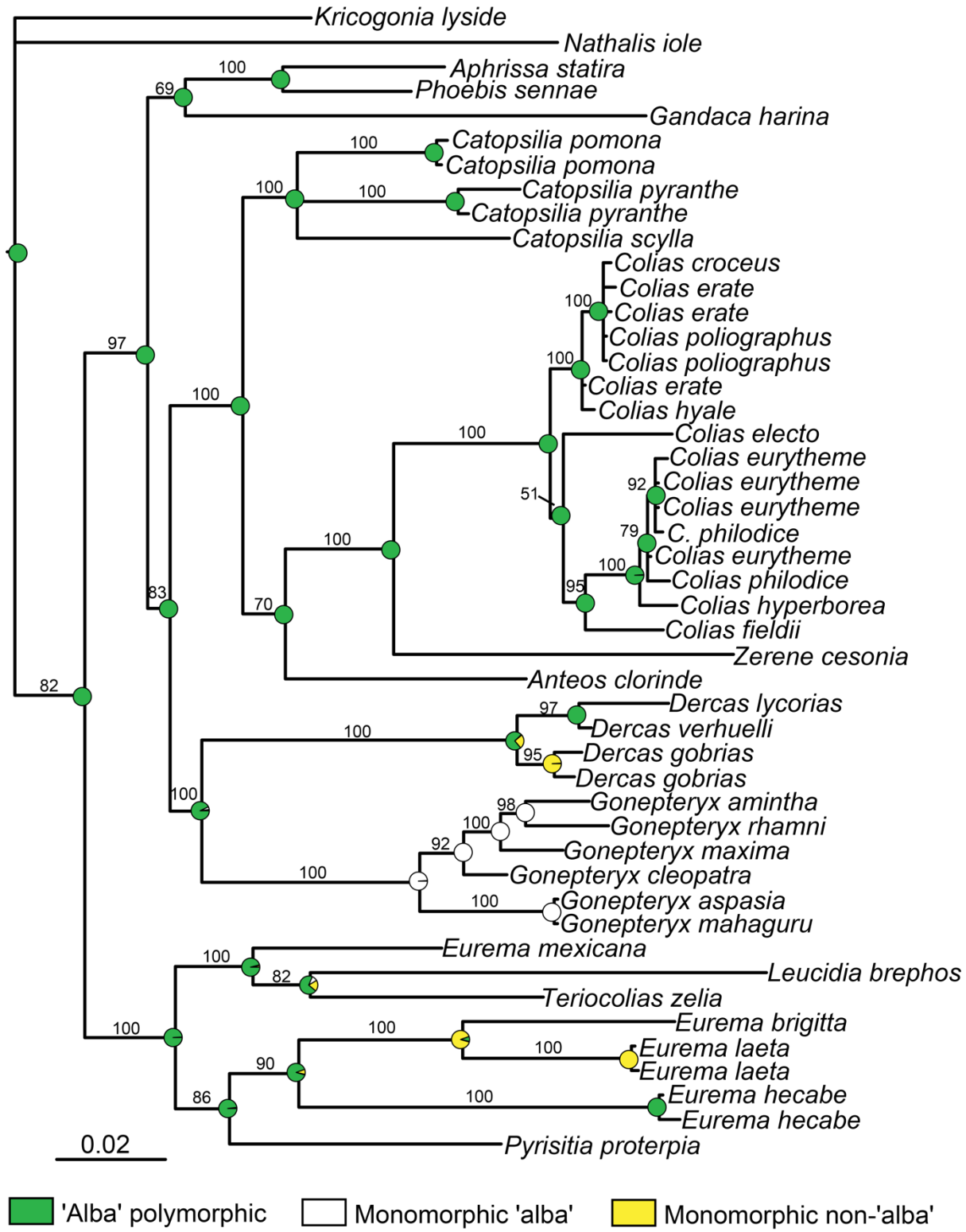

Figure 4. Molecular phylogeny ancestral state reconstruction

Molecular phylogeny ancestral state reconstruction performed on a phylogeny of 35 species generated by Bayesian analysis in MRBAYES.EXE (Ronquist & Huelsenbeck 2003). Model parameters are detailed in methods. Branch lengths represented by key at bottom of figure and posterior probabilities are noted above the branch. The area of the slices in the pie charts at internal nodes represent the probabilities of the character states for that ancestor.

This analysis also reveals with high certainty that the ancestor of the *Colias* was polymorphic. However, this analysis only included polymorphic *Colias* species due to the availability of genetic sequences. Therefore, conclusions drawn from this analysis about *Colias* should be interpreted with caution.

2.4 DISCUSSION

Herein we addressed hypotheses about the origin and evolutionary path of the 'alba' polymorphism within the Coliadinae. Our analyses revealed that the ancestral state of the Coliadinae is likely polymorphic, with two out of three of our phylogenetic approaches providing strong support for a polymorphic ancestral state. The species-level phylogeny yielded inconclusive assignment of the ancestral state. However, we note that this latter analysis was based on the least biologically realistic phylogeny. Because detailed species relationships within genera are not available, this analysis included all species in each genus condensed in a single large polytomy. Better species-level resolution would be valuable in improving both ancestral state estimates as well as the pattern of gains and losses of polymorphism within genera.

We also conclude that the ‘alba’ polymorphism is relatively unstable, because all three analyses show numerous monomorphic species that are phylogenetically dispersed. This indicates that the ‘alba’ polymorphism has been lost repeatedly. Though species relationships within genera are not defined, many genera, especially the large ones (e.g. *Colias* and *Eurema*), have monomorphic species of both forms. This indicates that there could be multiple character state transitions within genera, although more precise characterization of the evolutionary history of these events cannot be provided without more complete information about species-level phylogenetic relationships.

Colias is the largest genus in the Coliadinae, geographically widespread, and phenotypically diverse (it contains species of all 3 character states). Presumably for these reasons, it is the most thoroughly studied genus in the Coliadinae (Graham *et al.* 1980; Nielsen & Watt 2000; Grieshuber *et al.* 2012). All three analyses indicate that the ancestor of the genus *Colias* was polymorphic. The species relationships are not thoroughly resolved, but the high proportion of monomorphic species within the *Colias* suggests repeated losses of polymorphism. Furthermore, reversions to monomorphism involve the loss of both morph types (‘alba’ and non-‘alba’) at relatively equal frequencies. This indicates that morph fixation may be a random event, or that selective pressures do not consistently favor one morph over the other. This finding is in contrast to analyses of other groups, such as side-blotched lizards, where it was found that one morph was consistently lost, presumably due to consistent selective pressure acting against that morph (Corl *et al.* 2010).

The finding that the ‘alba’ polymorphism is likely the result of a single evolutionary origin may have significant implications for understanding its genetic basis. The ‘alba’ allele is thought to be a single, autosomal locus dictated by Mendelian genetics (Gerould 1923; Komai & Ae 1953). Future studies could look at whether genetic regulation of this locus is conserved across the Coliadinae.

Our comparative analysis of the evolutionary history of the 'alba' polymorphism may also help future efforts to parse amongst potential selective mechanisms favoring the maintenance and/or loss of this polymorphism. Numerous different mechanisms may maintain polymorphisms within a population, such as apostatic selection (Bond 2007), variation over space and/or time (Gray & McKinnon 2007), and countervailing selection (Nielsen & Watt 2000). These mechanisms may also vary in their stability over evolutionary time (Haldane & Jayakar 1963; Gray & McKinnon 2007). The knowledge that the ‘alba’ polymorphism is relatively unstable may provide preliminary clues to aid future studies investigating the selective pressures maintaining the polymorphism. For example, future studies could focus on investigating selective pressures that are less stable or consistent over evolutionary time (i.e. environmental factors).

A recent review on polymorphisms suggested that future directions for the field should test the hypothesis that polymorphisms promote speciation (McLean & Stuart-Fox 2014). Conversely, it is also possible that apostatic selection may constrain population divergence and hence speciation by maintaining polymorphism across multiple populations (Svensson, Abbott & Hardling 2005). One prediction of the hypothesis that polymorphism promotes speciation is that polymorphic taxa should be ancestral to monomorphic taxa because speciation of polymorphic ancestors would lead

to monomorphic daughter species (McLean & Stuart-Fox 2014). Phylogenies showing polymorphic ancestors resulting in many monomorphic descendant species would be consistent with this hypothesis whereas phylogenies showing monomorphic ancestors with polymorphic descendants would refute this hypothesis. Our data are consistent with the former, offering implicit support for the hypothesis that polymorphism may promote speciation.

Our study represents the first evaluation of the origin and history of the ubiquitous ‘alba’ polymorphism within the butterfly sub-family Coliadinae. The ‘alba’ polymorphism has been of great interest to researchers in diverse fields such as enzymatics (Watt 1977), thermoregulation (Kingsolver 1983), and mate choice (Gilchrist & Rutowski 1986; Kemp & Macedonia 2006; Limeri & Morehouse 2014). Our findings that the ‘alba’ polymorphism is likely ancestral and unstable is key in enabling future studies to explore the causes and mechanisms contributing to the maintenance of the ‘alba’ polymorphism. Because the ‘alba’ polymorphism is likely ancestral to both groups, pertinent questions may focus on why the polymorphism is maintained and what factors lead to the loss of either morph rather than the factors that lead to repeated origins of the polymorphism. Understanding the environmental drivers of the polymorphism is important, but more natural history information is a prerequisite. With improved natural history information, future studies could search for correlations between environmental factors (i.e. climate, host plant, co-ocurring species) and the maintenance or loss of polymorphism.

2.5 CONCLUSION

Understanding the evolutionary history of this trait is an important first step to characterizing the unique selective pressures acting to maintain a widespread color polymorphism. Here we conclude that the ‘alba’ polymorphism is ancestral to the Coliadinae and evolutionarily unstable. Further hypotheses should address questions related to the maintenance and repeated loss of this polymorphism.

2.6 ACKNOWLEDGEMENTS

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3.0 SENSORY LIMITATIONS AND THE MAINTENANCE OF COLOR POLYMORPHISMS: VIEWING THE 'ALBA' FEMALE POLYMORPHISM THROUGH THE VISUAL SYSTEM OF MALE *COLIAS* BUTTERFLIES

3.1 INTRODUCTION

Polymorphisms are not only fascinating biological phenomena; they also play a critical role in major evolutionary and ecological processes, including the formation of new species, intraspecific competition, and range expansion and contraction (Forsman *et al.* 2008). Color polymorphisms in particular are tractable phenomena for studying the evolution of polymorphisms because they are easily quantified and are common to many taxa (McKinnon & Pierotti 2010). Color polymorphisms have been implicated in sexual selection and predation avoidance in poison arrow frogs (Siddiqi *et al.* 2004), speciation in *Drosophila* (Wittkopp *et al.* 2009) and *Heliconius* butterflies (Kronforst *et al.* 2006; Chamberlain *et al.* 2009) and assortative mating in lesser snow geese (Cooke & McNally 1975). The persistence of polymorphisms presents an evolutionary paradox because it suggests that multiple morphs maintain roughly equivalent fitness over long periods of time. However, in many cases, little is known about the selective pressures that impact the fitness of each morph, or how these selective forces are balanced across evolutionary timescales.

One intriguing possibility is that color polymorphisms persist because they exploit perceptual limitations or biases of receivers, particularly mate-searching conspecifics. A

well-known example of this phenomenon is the existence of andromorph females in polymorphic damselflies (Sherratt 2008). Andromorph females are thought to visually mimic male damselflies and therefore benefit by avoiding costly male harassment (van Gossum, Stoks & De Bruyn 2001). This benefit is counterbalanced by decreased andromorph mating rates and potentially increased predation risk of these more brightly colored andromorph females in comparison to their more cryptically colored gynomorph female conspecifics (Robertson 1985; van Gossum *et al.* 2001; Sirot *et al.* 2003). Similar, although less extensively-studied examples can be found in other animal taxa. For instance, the Mocker Swallowtail butterfly, *Papilio dardanus*, possesses as many as 14 female-limited color morphs (Nijhout 2003). Most of these morphs mimic different distasteful butterflies, thereby gaining protection from predation as Batesian mimics (Nijhout 2003). However, one *P. dardanus* female morph resembles the male visual phenotype. Similar to andromorph damselflies, these female butterflies are thought to mimic male coloration in order to avoid male courtship harassment (Cook *et al.* 1994). Polymorphism in *P. dardanus* thus appears to be maintained, at least in part, via exploitation of characteristics of male visual mate recognition.

Although limitations to male mate recognition appear to be important for the evolutionary maintenance of color polymorphisms in a range of taxa, and theoretical treatments suggest that such scenarios are likely to be of widespread importance (Chunco, McKinnon & Servedio 2007), researchers have typically relied on human visual assessments to identify color morphs that should present challenges for mate searching males. Such assessments have limited value because human color vision is often dramatically different from that of focal research taxa (Kelber 2006; Land &

Nilsson 2012). Thus, the more pertinent (and as yet unanswered) question is whether female morphs present discrimination challenges when viewed through the visual systems of male conspecifics. Rapid advances in vision research and visual ecology over the past several decades now enable visual system modeling for a growing number of animal species (Kelber & Osorio 2010). However, these techniques have yet to be applied to systems where males must discriminate female morphs from other similar but reproductively unprofitable visual stimuli.

We pursued this opportunity using color measurements and visual system modeling for polymorphic butterfly species in the genus *Colias.* This group is characterized by a ubiquitous, female-limited, wing color polymorphism, called the 'alba' polymorphism, with at least 51 out of 79 species in the genus exhibiting 'alba' female morphs (Limeri, unpublished data). Female wings are either colored similarly to males, which are yellow or orange with black borders, or they are pale white with black borders (a form termed 'alba'). Wing coloration differences between female color morphs are genetically determined by a dominant autosomal locus (Lorkovic & Herman 1961). 'Alba' females are thought to enjoy a metabolic advantage over non-'alba' females because they do not invest in costly yellow pigmentation, resulting in higher early-life fecundity (Graham *et al.* 1980). However, researchers have suggested that this fecundity benefit is counteracted by a male mating bias against 'alba' females, leading to reduced mating opportunities and concurrent reductions in overall fitness (Watt 1995).

The argument for why males might discriminate against 'alba' females despite their higher fecundity relies on the idea that males should experience difficulties in

identifying conspecific ‘alba’ females from other similarly-colored heterospecific females. *Colias* butterflies co-occur with a number of other butterfly species that resemble the ‘alba’ phenotype, at least to the human eye, including white butterflies in the sub-family Pierinae and the ‘alba’ females of other co-flying *Colias* butterflies. This similarity in appearance is relevant to mate selection because *Colias* males first identify mates from a distance using visual cues (Silberglied & Taylor 1978). Misdirected mating attempts should result in non-trivial fitness costs for males, ranging from losses of search time and energy to more substantial costs associated with hybrid matings (Ae 1959; Grula & Taylor 1980). The expectation is that these costs should result in a male preference bias against ‘alba’ females, either due to negative reinforcement based on male experiences during mate searching or the evolution of genetically-determined reductions in male interest in ‘alba’-like stimuli.

Behavioral evidence for a male mating bias against ‘alba’ females has been ambiguous to date. While some studies have reported data suggesting that males discriminate against ‘alba’ females (Graham *et al.* 1980; Watt 1995), others have gathered neutral or contradicting evidence, in some cases reporting higher mating success for ‘alba’ females (Gilchrist & Rutowski 1986; Nakanishi, Watanabe & Ito 2000; Kemp & Macedonia 2006). These contradictory results suggest that we do not fully understand the determinants of male mate choice in relation to the ‘alba’ morph in *Colias* butterflies. In particular, we still do not know whether males should face challenges in visually discriminating ‘alba’ females from other reproductively unprofitable butterflies typically encountered in their environment. If such limitations to male mate recognition exist, then the observed variation in ‘alba’ mating success may

reflect population-specific differences in the risk of misidentifying ‘alba’ females (i.e., due to higher or lower population densities of co-occurring white heterospecific butterflies).

We tested three related but not mutually-exclusive hypotheses concerning the role that male visual discrimination may play in driving patterns of mating success for ‘alba’ versus non-‘alba’ female phenotypes. First, following Nielsen and Watt (2000), we tested the hypothesis that males may face challenges visually discriminating ‘alba’ females for co-occurring heterospecific (and therefore reproductively non-valuable) white butterflies, particularly members of the ubiquitous genus *Pieris*. Alternatively, we hypothesized that males may have a difficult time visually distinguishing between conspecific and heterospecific ‘alba’ females where multiple *Colias* species coexist. If males do visually mistake conspecific ‘alba’ females for pierids or heterospecific ‘alba’ females, reproductive interference could result that may cause males to respond differently to ‘alba’ females depending on community composition. Lastly, we tested the hypothesis that ‘alba’ and non-‘alba’ females differ in their visual salience, either due to differences in brightness or color contrast from the plant foliage against which they are typically viewed. Such differences in salience may result in different levels of male courtship attention focused on these two female color morphs, leading to divergent mating rates for ‘alba’ and non-‘alba’ females.

We tested these three hypotheses in a community of co-flying butterflies where two *Colias* species exhibiting 'alba' polymorphisms (*Colias philodice* Godart and *Colias eurytheme* Boisduval, Fig. 5) co-occur alongside several similarly-sized white butterflies, including the common butterfly *Pieris rapae* L. (Fig. 5). In western Pennsylvania, *C.*

philodice and *C. eurytheme* are found in the same habitats across the growing season, and appear to hybridize frequently despite documented costs associated with hybrid matings (Ae 1959). To formally test our three proposed hypotheses, we measured wing reflectance from *C. philodice*, *C. eurytheme* and *P. rapae*. We then constructed mathematical models of color visual discrimination using data from the closely-related congener *C. erate* to estimate the likelihood that the 'alba' female phenotype presents a challenge for the male visual system to distinguish from 1) white heterospecifics, 2) congeneric 'alba' females or 3) foliage backgrounds.

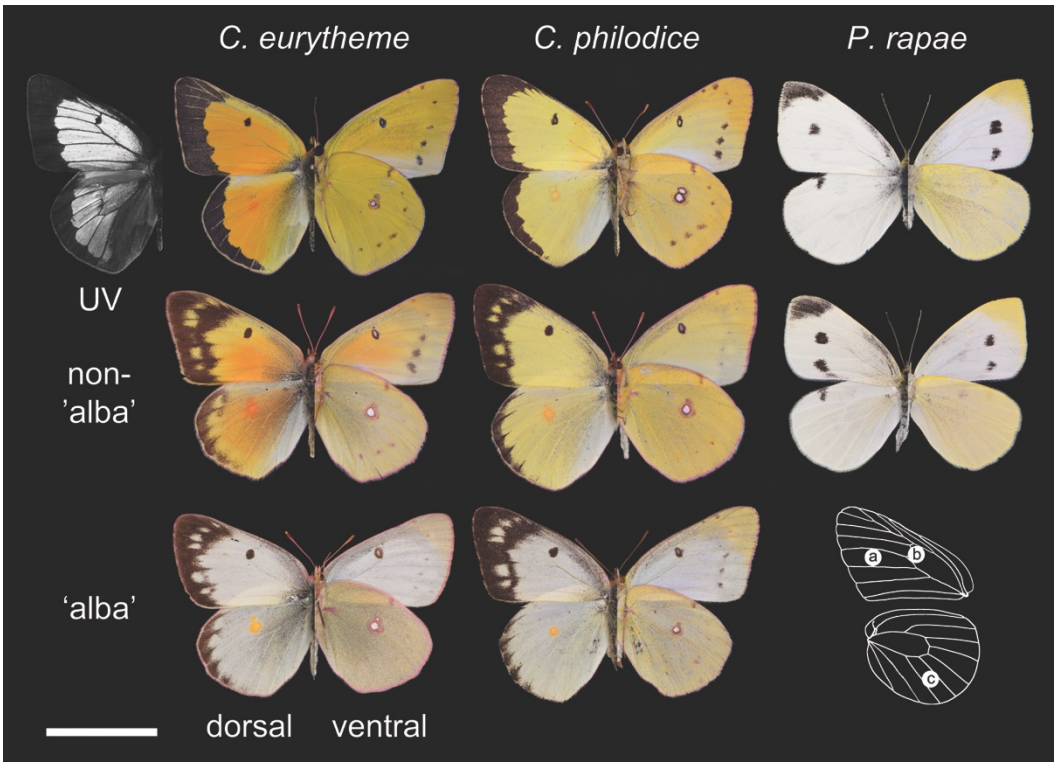

Figure 5. Images of the focal study species

Images of focal study species, including *C. eurytheme* (left), *C. philodice* (center) and *P. rapae* (right). Males appear in the top row with females below (for *Colias* species, 'alba' females are both in the bottom

row). The left side of each butterfly illustrates the dorsal wing surface and the right side illustrates the ventral wing surface. The dorsal surface of male *C. eurytheme* is also shown imaged using only ultraviolet light to illustrate male-limited UV coloration (UV, far left). White circles in the wing venation diagrams at bottom right (after Scott 1986) indicate where spectral measurements were taken on the dorsal forewing of *Colias* species (a) and *P. rapae* (b), and for the ventral hindwing surface of all species (c). Scale bar on bottom left is 2 cm.

3.2 MATERIALS AND METHODS

3.2.1 Rearing

C. philodice females (n = 5) and *C. eurytheme* females (n = 6) were caught in agricultural alfalfa fields in Rochester, PA, USA (40.745664°N, 80.163377°W) and brought into the lab to lay eggs. Parental *C. philodice* females were a mix of homozygous (n = 2) and heterozygous (n = 3) 'alba' morphs, with the latter females producing both non-'alba' and 'alba' female offspring. *C. eurytheme* parental females included non-'alba' females (n = 3) as well as homozygous (n = 1) and heterozygous (n = 2) 'alba' females. In combination, these females likewise produced a mixture of 'alba' and non-'alba' female offspring. Caterpillars were raised on alfalfa (*Medicago sativa* L.) in climate-controlled chambers that maintained a coincident photoperiod and temperature cycle (16:8, light:dark, 30°C:24°C) with a constant vapor pressure deficit of 1.7 kPa. Alfalfa was harvested from the same field that the butterflies were collected in and surface sterilized to prevent disease introduction into the rearing program by

soaking in dilute bleach water for 30 minutes followed by rinsing in water. Upon eclosion and following wing hardening, males and females from each family were freeze euthanized to preserve wing coloration. Additionally, we reared *P. rapae* on lab-grown kale (*Brassica oleracea* L.) in the same climate chamber and freeze euthanized freshly eclosed adults for later color measurements.

We chose to measure coloration of lab-reared individuals rather than from field-caught butterflies for two reasons. First, characters for reliably discriminating the 'alba' females of *C. eurytheme* and *C. philodice* have not been reported to date. Thus, lab-rearing was required to unequivocally assign species identity to these butterflies. Second, as butterflies age, the scales that color their wings are slowly lost. This gradual but somewhat stochastic process of wing wear makes comparisons between field-caught individuals of varying ages considerably more complex. We chose to control for this source of variation by focusing our measurements on fresh lab-reared individuals. However, we consider the relevance of age-related wing color fading to our results in the discussion below.

3.2.2 Wing color measurements

Sample sizes for wing reflectance measurements are as follows: *C. eurytheme* males (n = 11), 'alba' females (n = 8), yellow females (n = 10); *C. philodice* males (n = 12), 'alba' females (n = 11), yellow females (n = 8); *P. rapae* males (n = 10), and females (n = 10). Wing color measurements were taken following previously described methods (Morehouse & Rutowski 2010). Briefly, we removed wings from freshly

eclosed, freeze-euthanized specimens and mounted a single forewing with the dorsal surface facing up and a single hindwing with the ventral surface facing up on matte black cardstock using archival spray adhesive (Scotch® Photo Mount, 3M, St. Paul, MN). Measurement locations were selected to best represent the dominant color of the wing surface (Fig. 5). For *Colias*, we measured the center of the M3 cell (wing cell identities and naming conventions are dictated by wing venation patterns, Scott 1986) of the dorsal forewing surface and directly below the discal cell in the CuA_1 cell of the ventral hindwing surface (Fig. 5). *C. eurytheme* males exhibit structurally-produced, iridescent ultraviolet (UV) patterns on the dorsal surfaces of their wings (Fig. 5, Rutowski *et al.* 2007). Light reflectance by these UV colors is highly directional such that their visibility is dependent on the geometry of incident light, wing surface and viewer. Thus these brilliant iridescent UV patterns may often but not always contribute to the color seen by mate-searching males (Rutowski *et al.* 2007). Therefore, we measured male *C. eurytheme* forewings in the same orientation as other wing samples (the "UV-visible" orientation) and rotated 90^{o} counter-clockwise (the "UV-absent" orientation) to capture the range of male appearances during flight in the field. For *P. rapae*, the location of the black spots on the forewing made it difficult to take measurements in the same place as for *Colias*. Measurements were therefore taken at the distal tip of the discal cell on the forewing and the center of the CuA_1 cell of the hindwing (Fig. 5).

We collected reflectance spectra in a dark room using a spectrophotometer (USB4000, Ocean Optics, Dunedin, FL) calibrated with a magnesium oxide white standard. Wing surfaces were illuminated using a deuterium-tungsten-halogen light source (DH2000-BAL, Ocean Optics, Dunedin, FL) positioned normal to the wing

surface at the zenith above the mounted wings. Light reflected off the wing surfaces was sampled using a collimated fiber optic collector positioned at 45^{o} below the zenith toward the proximal wing margin along the distal-proximal axis of the wing, with the exception of the “UV-absent” orientation (see above), where the collector was positioned above the posterior margin of the wing along the anterior-posterior wing axis. Measurements were recorded as percent reflectance from 300-800 nm (Fig. 6).

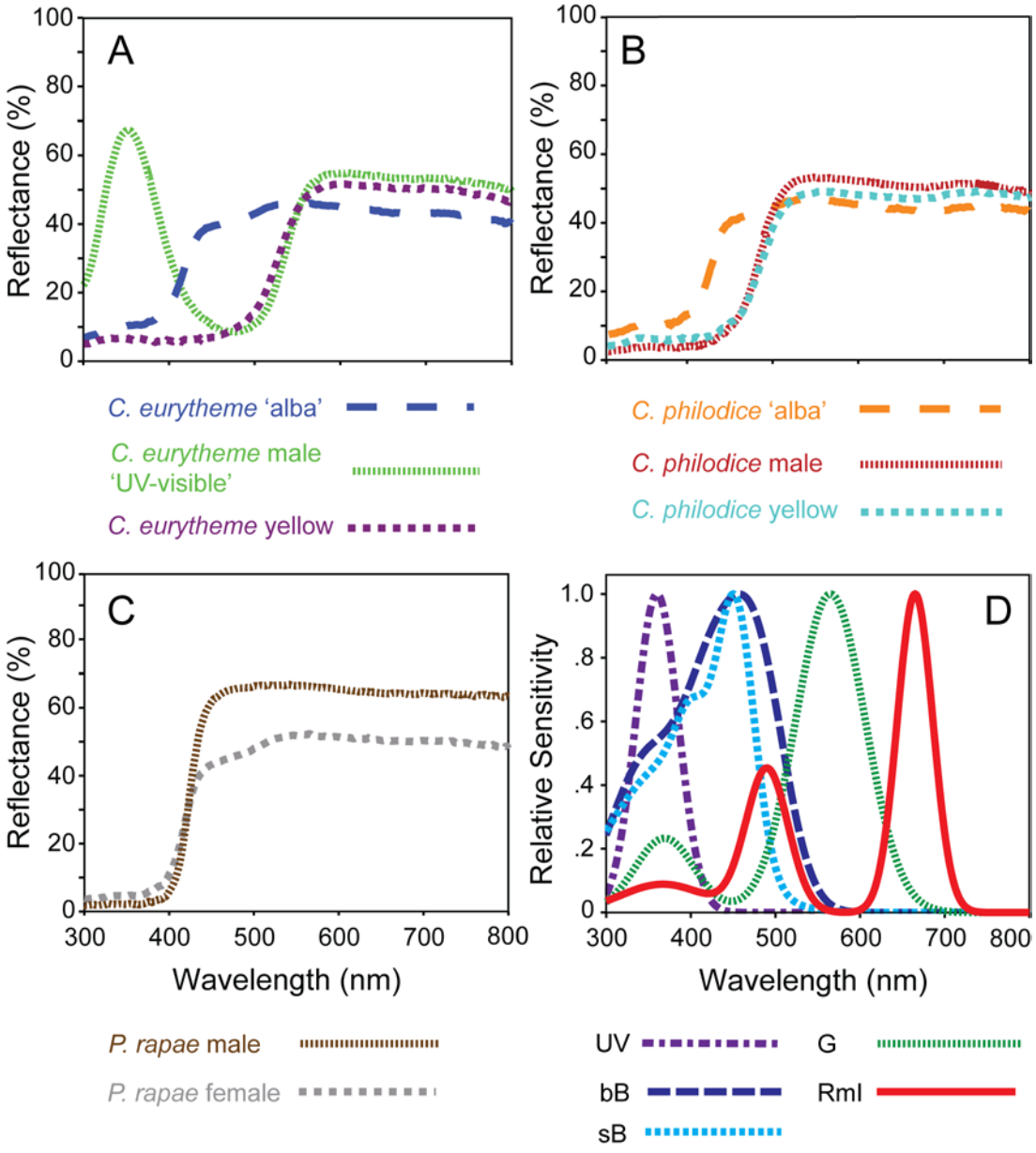

Figure 6. Average reflectance spectra

Average reflectance spectra for *C. eurytheme* males, 'alba' females, and yellow females (A), *C. philodice* males, 'alba' females, and yellow females (B), and *P. rapae* males and females (C). Normalized *C. erate* photoreceptor sensitivity curves for the five photoreceptors used in the visual system model are presented in (D).

3.2.3 Visual system model

We constructed a receptor noise-limited color opponency model based on methods originally developed by Vorobyev and Osorio (1998). The best information to date suggests that *Colias* species are likely to be pentachromatic despite the fact that their eyes contain 8 distinct photoreceptor types (Ogawa et al. 2012; Ogawa et al. 2013). We therefore extended the Vorobyev and Osorio (1998) model to pentachromacy following formulae from Morehouse and Rutowski (2010) as described below.

Both vertebrates and invertebrates interpret light reflected from surfaces using chromatic and achromatic (luminance) contrast separately (Kelber, Vorobyev & Osorio 2003; Endler & Mielke 2005). The following equation, adapted from Morehouse and Rutowski (2010), was used to calculate chromatic contrast (contrast with background; ΔS) and color discriminability (chromatic contrast between two focal stimuli; ΔS^t):

$$\begin{aligned}\Delta S^{\mathrm{t}} \text{or } \Delta S = \{&[(e_3e_4e_5)^2(\Delta q_1 - \Delta q_2)^2 + (e_2e_4e_5)^2(\Delta q_1 - \Delta q_3)^2 + (e_2e_3e_5)^2(\Delta q_1 - \Delta q_4)^2 \\ &+ (e_2e_3e_4)^2(\Delta q_1 - \Delta q_5)^2 + (e_1e_4e_5)^2(\Delta q_2 - \Delta q_3)^2 + (e_1e_3e_5)^2(\Delta q_2 - \Delta q_4)^2 \\ &+ (e_1e_3e_4)^2(\Delta q_2 - \Delta q_5)^2 + (e_1e_2e_5)^2(\Delta q_3 - \Delta q_4)^2 + (e_1e_2e_4)^2(\Delta q_3 - \Delta q_5)^2 \\ &+ (e_1e_2e_3)^2(\Delta q_4 - \Delta q_5)^2\Big] \\ &/[(e_1e_2e_3e_4)^2 + (e_1e_2e_3e_5)^2 + (e_1e_2e_4e_5)^2 + (e_1e_3e_4e_5)^2 \\ &+ (e_2e_3e_4e_5)^2\,]\Big\}^{-1/2}\end{aligned}$$

For ΔS^t, Δq_i represents difference in photoreceptor output between two stimuli (e.g., q_i^1 and q_i^2). For ΔS, Δq_i is the difference in photoreceptor output between the focal stimulus (q_i) and the foliar background (q_i^B), which mathematically simplifies to q_i because q_i^B reduces to zero during von Kries transformation (see below). q_i was determined using the following equation representing a von Kries transformation of photoreceptor quantum catches:

$$q_i = \ln(\frac{Q_i}{Q_i^B})$$

where

$$Q_i = \int_{300}^{800} R(\lambda)I(\lambda)A_i(\lambda)d\lambda$$

$$Q_i^B = \int_{300}^{800} R^B(\lambda)I(\lambda)A_i(\lambda)d\lambda$$

and λ is wavelength (nm), $R(\lambda)$ is the reflectance of a color stimulus at a given wavelength, $R^B(\lambda)$ is the reflectance of the background, $I(\lambda)$ is the ambient irradiance in units of photon flux (μmol m^{-2} s^{-1} nm^{-1}), and $A_i(\lambda)$ is the wavelength-specific absorbance of photoreceptor *i*.

In contemporary populations, interactions between males and females occur most frequently in agricultural monocultures of their host plant alfalfa (*M. sativa*), where males are found searching for recently eclosed virgin females or previously-mated yet

receptive females. We therefore characterized background reflectance (R^B) as the average reflectance spectra from five alfalfa (*M. sativa*) leaves collected from the same field sites as our focal butterflies. In the populations we studied, *Colias* butterfly activity is concentrated around midday, with much higher levels of activity on sunny days (Morehouse, personal observation). We thus collected irradiance (I) data at noon on a cloudless day in Pittsburgh, PA using an Ocean Optics JAZ spectrophotometer calibrated using a standard light source (LS-1 CAL, Ocean Optics, Dunedin, FL).

Photoreceptor sensitivity values (A_i) were generated from photoreceptor absorbance templates fit to intracellular recordings reported from a closely-related congener, *Colias erate* (Pirih, Arikawa & Stavenga 2010; Ogawa *et al.* 2012, 2013). Species-specific data are not available for *C. eurytheme* or *C. philodice*. Thus, spectral sensitivities from *C. erate* are the closest data available for approximating the spectral sensitivity of our focal *Colias* species. In support of the implicit assumption that visual sensitivities are conserved between *C. erate, C. eurytheme,* and *C. philodice*, we note that although visual sensitivities are evolutionarily labile across the Lepidoptera (Briscoe 2008), they also appear to exhibit relatively high levels of conservation between closely related taxa (e.g., within the Pieridae, Awata, Wakakuwa & Arikawa 2009). We also note that ‘alba’ females are found in *C. erate.* Nevertheless, we acknowledge that the use of visual system information from *C. erate* serves only as a first approximation for how the eyes of *C. eurytheme* and *C. philodice* might view conspecific and heterospecific coloration.

In male *C. erate*, eight photoreceptor types have been characterized from the eye: ultraviolet (UV), shouldered blue (sB), broad blue (bB), broad green (bG), green

(G), red male type I (RmI), red male type II (RmII), and red male type III (RmIII) (Pirih *et al.* 2010; Ogawa *et al.* 2012, 2013). We selected a subset of five for inclusion in our visual system model: UV, sB, bB, G, and RmI (Fig. 6D). We excluded bG because it is a rare subclass of green receptors unlikely to contribute to color vision beyond the inputs of the much more common G receptors (K. Arikawa, pers. comm.). RmII and RmIII were excluded because they exhibit large secondary sensitivity peaks in the short wavelengths, making them less useful for color vision (K. Arikawa, pers. comm.).

Receptor noise values (e_i) were calculated by dividing the Weber fraction by the square root of average photoreceptor abundances within a single ommatidium. This method assumes that effective photoreceptor noise within each color-sensitive channel is reduced via summation within individual ommatidia, an assumption supported by behavioral data in honeybees (Vorobyev et al. 2001). We chose a Weber fraction of 0.01 based on empirical estimates from the butterfly *Papilio xuthus* (Koshitaka *et al.* 2008), the only butterfly species for which such estimates are available. Photoreceptor abundances were obtained from prior histological characterization of ommatidial types within the eye of male *C. erate* (Awata *et al.* 2009).

We calculated values for ΔS (chromatic contrast against background) for each wing color measurement for each group. We then calculated ΔS^t (chromatic contrast between two stimuli) for all possible pairwise comparisons between 8 individuals per group (a total of 64 comparisons per wing color measurement per group pair). This method of calculating ΔS^t allowed us to estimate both mean ΔS^t and its variance for each comparison. However, because the resulting set of ΔS^t values within a specific comparison are not independent from each other (i.e., they are generated by a shared

set of original values), our statistical analysis of ΔS^t is restricted to qualitative comparisons of mean ΔS^t values as well as formal evaluation of ΔS^t overlap with specific numerical thresholds (see below).Values for ΔS and ΔS^t calculated using our visual system model are estimates of the discriminability of focal stimuli, scaled in units of standard deviations of photoreceptor noise. These discriminability values can be interpreted in two ways, either against an absolute threshold (the "just noticeable difference" or jnd) or in relative terms. For the former interpretation, individuals should theoretically be able to discriminate stimuli that differ by a ΔS^t value of only 1. However, behavioral estimates derived from research using the European honeybee (*Apis mellifera*, Giurfa *et al.* 1997) suggest that a value of 2.3 is more likely to represent the threshold above which color-based decisions can be reliably made (>75% accuracy) when stimuli are presented side-by-side. We therefore interpret all stimuli estimated to have a ΔS^t value of larger than 2.3 to be potentially discriminable by the male *Colias* eye, at least when presented simultaneously.

Discriminability values can also be considered relative to one another, with groups exhibiting lower discriminability values considered more difficult for male *Colias* to distinguish from each other. This latter method of interpretation is more likely to provide valuable insight into color discrimination when color stimuli are experienced at greater distances in time or space (i.e., not simultaneously) where errors introduced via processes of stimuli generalization and memory may substantially increase discrimination thresholds above the values considered reasonable for side-by-side comparison of stimuli (Kelber *et al.* 2003).

The five photoreceptors identified above are those most likely to be involved in chromatic discrimination, but it is unknown which photoreceptors participate in achromatic visual discrimination in *Colias*. Many species of bees, including bumblebees (*Bombus impatiens*) and honeybees (*Apis mellifera*), use only their green photoreceptors to discriminate luminance (Giurfa & Vorobyev 1998; Hempel de Ibarra, Giurfa & Vorobyev 2002). However, recent work demonstrates that *Papilio* butterflies use the same photoreceptors for chromatic vision as for luminance discrimination (Koshitaka, Arikawa & Kinoshita 2011). We therefore chose to calculate luminance contrast scores that include input from all five photoreceptors as follows:

$$L = \sum_{i=1}^{N} \alpha_i q_i$$

where

$$\alpha_i = \frac{\eta_i}{\eta}$$

The parameter α_i weights the contribution of photoreceptor *i* to luminance contrast on the basis of its abundance in the eye (η_i) relative to the total number of photoreceptors (η). Rules of thumb relating achromatic contrast values to behavioral decision-making thresholds are lacking for this method of estimating achromatic contrast. Therefore, *L* values estimated to overlap with zero are considered non-discriminable, but larger estimates are discussed as potentially discriminable. We calculated luminance contrast against the foliage background as well as between the wing colors of all groups. For the latter comparisons, as with estimates of ΔS^t, we

compared all possible pairwise differences for each wing color measurement for 8 individuals per group (a total of 64 comparisons per wing color measurement contrast for each group comparison).

We note that in general, luminance differences between stimuli are thought to be less reliable in terrestrial environments than chromatic contrast cues because of high temporal and spatial heterogeneity in irradiance (Kelber *et al.* 2003). Nevertheless, achromatic contrast does present a source of visual information that mate-searching *Colias* males may employ when identifying potential mates. In particular, achromatic contrasts with background foliage may be relevant as wing phenotypes that exhibit higher achromatic contrasts with background are likely to be more salient across a range of illumination scenarios.

3.2.4 Statistical analyses

All statistical analyses detailed below were performed using SPSS 20 (IBM Corp., Armonk, NY). Data normality and homoscedasticity were verified prior to running statistical tests. A one-way ANOVA was performed for chromatic (ΔS) and luminance contrasts (L) between the wing and the background. Post-hoc comparisons were conducted using the Tukey-Kramer method to maintain an experiment-wise $\alpha$of 0.05. For chromatic (ΔS^t) and luminance contrasts (L) between groups, formal statistical analyses were precluded because values within these datasets were non-independent (see above). However, we were able to qualitatively compare mean values for these metrics. In addition, we calculated 95% confidence intervals for both ΔS^t and L, and

evaluated them for overlap with the critical thresholds identified above (2.3 for chromatic contrast and 0 for luminance contrast).

3.3 RESULTS

3.3.1 Wing contrast with foliar background

First, we compared chromatic contrasts between each of our focal wing colors and the foliar background against which they would typically be viewed. These values estimate a male's ability to locate a single individual within the environment using chromatic cues, and present a specific test of our hypothesis that 'alba' females differ from non-'alba' female morphs in their salience in the field. All estimates were well above the 2.3 threshold, indicating that male *Colias* should be able to readily discriminate all of the focal wing colors from the background. However, comparison between groups revealed significant differences in chromatic contrast to background (forewing chromatic contrast: $F_{8,82} = 64.1$, $p < 0.01$; hindwing chromatic contrast: $F_{7,72} = 90.8$, $p < 0.01$; Table 1). For both hindwing and forewing chromatic contrast comparisons, *C. eurytheme* and *C. philodice* 'alba' females were not statistically different from each other and differed from all other groups (Fig. 7A,B). These 'alba' forms exhibited the lowest chromatic contrast scores against foliage backgrounds of any wing colors considered in this study. We also observed a general pattern of reduced chromatic contrast against background for ventral hindwing colors as compared to dorsal forewing colors, with more dramatic differences between dorsal and ventral wing

surfaces in females compared to males (Table 1, Figure 7A,B). The one exception to this pattern was observed for male *C. eurytheme* dorsal coloration in the "UV-visible" position, which exhibited lower chromatic contrast scores when compared to the ventral wing coloration in those individuals.

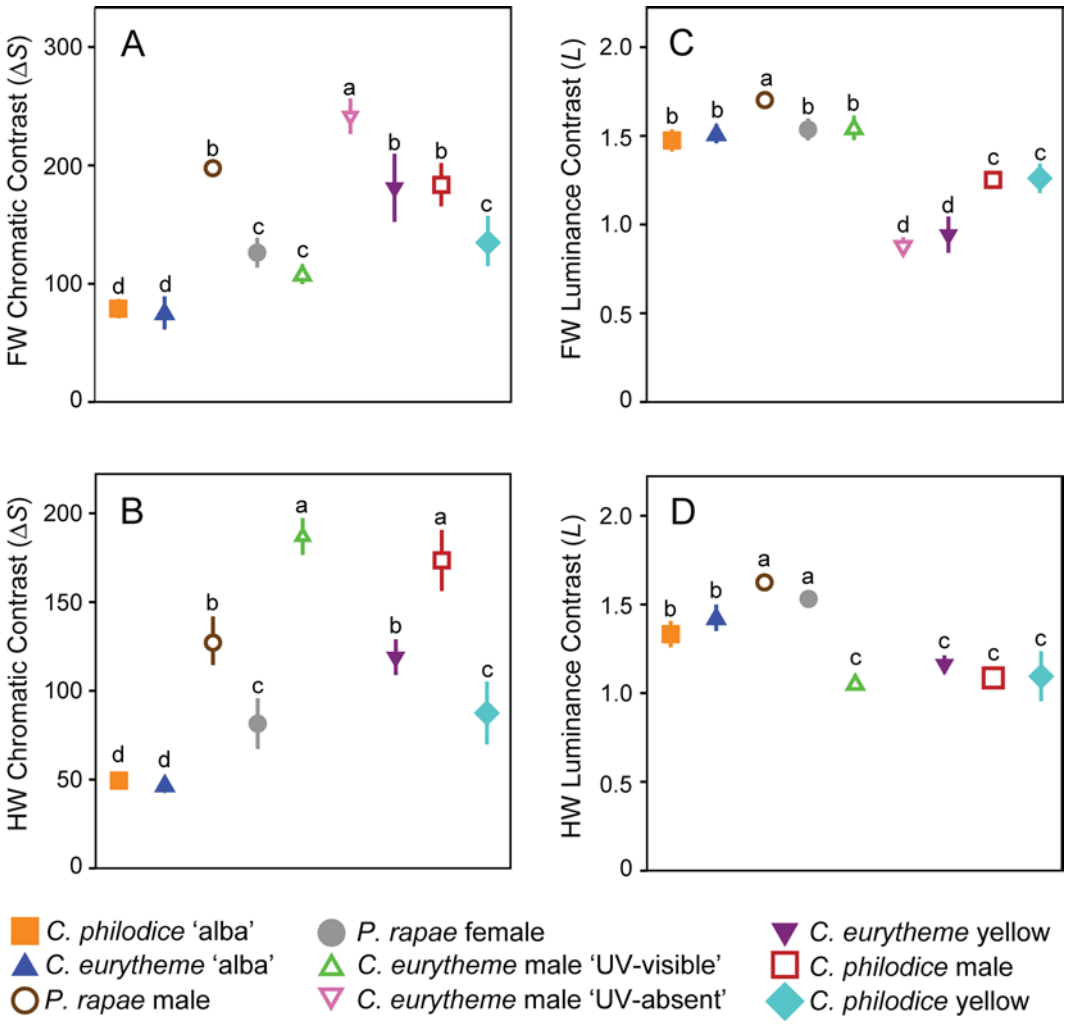

Figure 7. Chromatic and luminance contrasts between wing and foliar background

Chromatic and luminance contrasts between wing and foliar background of forewing (FW) chromatic contrasts (A), hindwing (HW) chromatic contrasts (B), forewing luminance contrasts (C), and hindwing luminance contrasts (D). Error bars represent 95% confidence intervals. For symbols where no error bar is visible, error bars are smaller than the size of the symbol itself. For luminance contrasts, a positive

value indicates that the butterfly wing is brighter than the background foliage. Groups with different letters are statistically distinguishable from one another.

Table 1. Chromatic and luminance contrasts between wing surfaces and background

Chromatic (ΔS) and luminance (L) contrasts (means ± confidence intervals) between wing coloration and background for all groups. For chromatic contrast, values above 2.3 are potentially discriminable under optimal conditions, and for luminance contrast, values that do not overlap with 0 are potentially discriminable.

Species	Sex/Morph	Forewing		Hindwing	
		ΔS	*L*	ΔS	*L*
C. eurytheme	'Alba'	75.26 ± 15.89	1.51 ± 0.06	46.98 ± 12.54	1.43 ± 0.06
	Non-'alba'	181.10 ± 14.22	0.94 ± 0.05	118.97 ± 11.22	1.16 ± 0.06
	"UV-visible" male	108.00 ± 13.55	1.55 ± 0.05	186.95 ± 10.70	1.06 ± 0.05
	"UV-absent" male	241.55 ± 13.55	0.88 ± 0.05		
C. philodice	'Alba'	81.00 ± 13.55	1.47 ± 0.05	49.43 ± 10.70	1.33 ± 0.05
	Non-'alba'	135.99 ± 15.89	1.26 ± 0.06	87.51 ± 12.54	1.10 ± 0.03
	Male	183.63 ± 12.98	1.25 ± 0.05	173.49 ± 10.24	1.09 ± 0.05
P. rapae	Female	126.21 ± 14.22	1.54 ± 0.05	81.52 ± 11.22	1.53 ± 0.06

	Male	197.60 ± 14.21	1.70 ± 0.05	128.21 ± 11.22	1.63 ± 0.06

Second, we compared luminance contrasts between each of our focal wing colors and the foliar background. These values estimate a male's ability to discriminate a focal individual against foliage using achromatic cues. All luminance contrasts were positive and significantly different from 0 (Table 1, Fig. 7C,D), indicating that male *Colias* should be able to readily discriminate all wings from the darker background foliage. Statistical comparisons between group means revealed significant differences in luminance contrast against background (forewing luminance contrast: $F_{8,82} = 121.0$, $p < 0.01$; hindwing luminance contrast $F_{7,72} = 60.3$, $p < 0.01$; Table 1). For luminance contrast comparisons of forewing and hindwing coloration, *C. eurytheme* and *C. philodice* 'alba' females were not statistically different from each other (Fig. 7C and D). For forewing luminance contrast, they also did not differ from *P. rapae* females and *C. eurytheme* males in the "UV-visible" orientation (Fig. 7C). 'Alba' female colors were also consistently high in luminance contrast, with higher forewing luminance contrast scores found only for *P. rapae* males. For hindwing luminance contrast, 'alba' females were statistically brighter than all other groups (Fig. 7D).

3.3.2 Visual contrasts between species wing colors

We performed chromatic and luminance contrast comparisons between the forewings and separately between the hindwings of all possible groups. These comparisons estimate the perceived differences in color or luminance between focal

stimuli (wing colors) if viewed side-by-side. Here, we report the full results from comparisons between *C. eurytheme* 'alba' females and all other groups (Table 2; Fig. 8). However, results from the same comparisons for *C. philodice* 'alba' females were qualitatively similar and are reported in full in the supplemental material (Table S1; Fig. S1).

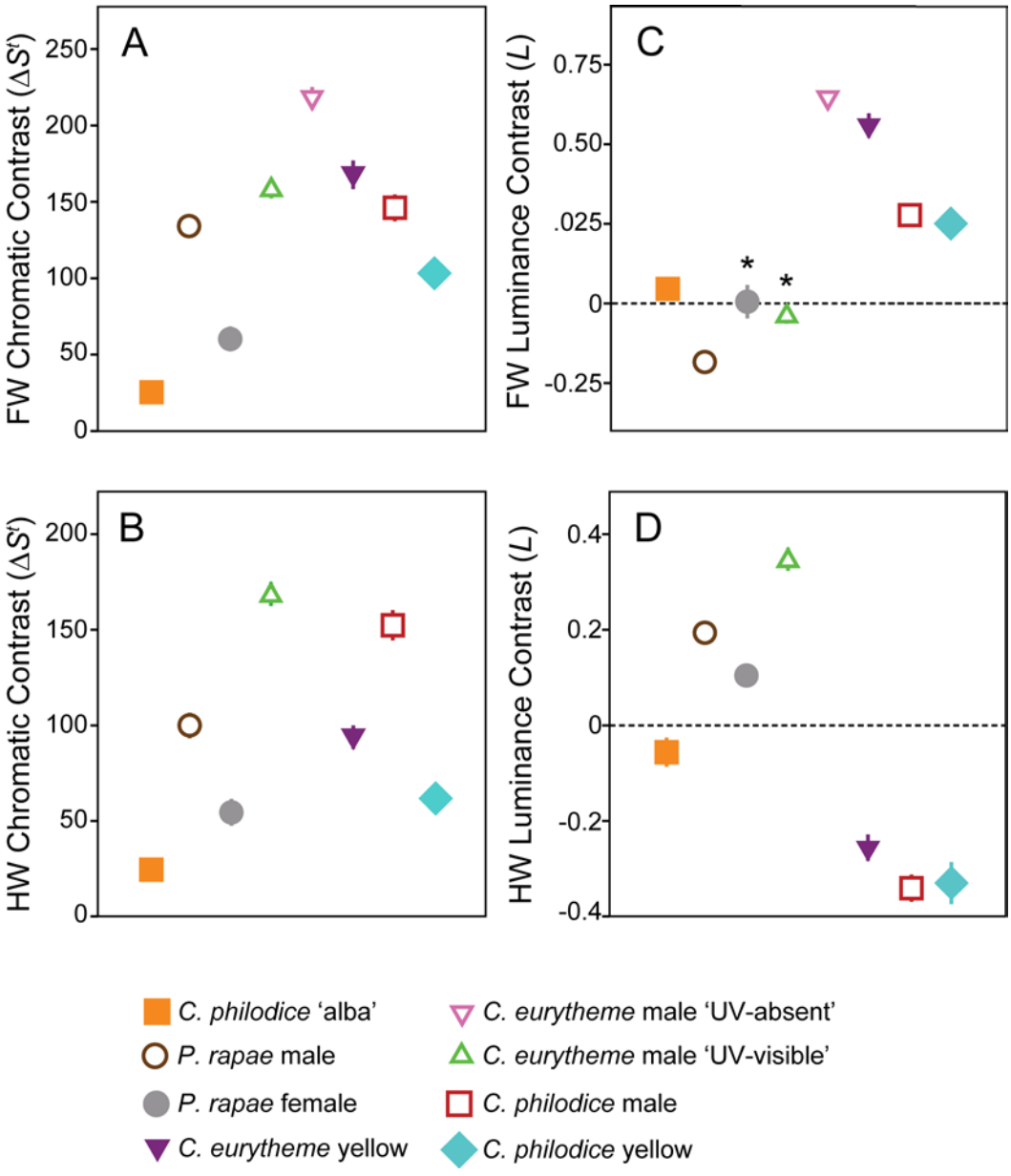

Figure 8. Comparisons between *C. eurytheme* 'alba' females and all other groups

Comparisons between *C. eurytheme* 'alba' females and all other groups for forewing (FW) chromatic contrast (A), hindwing (HW) chromatic contrast (B), forewing luminance contrast (C), and hindwing luminance contrast (D). Error bars represent 95% confidence intervals. For symbols where no error bar is visible, error bars are smaller than the size of the symbol itself. For luminance contrast, a positive mean indicates that *C. eurytheme* 'alba' females are brighter (have higher luminance) than the group coded by the symbol key. A negative mean indicates the opposite. Asterisks indicate groups that overlap with 0.

Table 2. Chromatic and luminance contrasts between *C. eurytheme* 'alba' females and all other groups.

Chromatic (ΔS^t) and luminance (L) contrasts (means ± confidence intervals) between *C. eurytheme* 'alba' females and all other groups. For chromatic contrast, values above 2.3 are potentially discriminable, and for luminance contrast, values that do not overlap with 0 are potentially discriminable. For luminance contrast, a positive mean indicates that *C. eurytheme* 'alba' females are brighter (have higher luminance) than the other group being compared.

Species	Sex/Morph	Forewing		Hindwing	
		ΔS^t	L	ΔS^t	L
C. eurytheme	Non-'alba'	167.69 ± 9.40	0.56 ± 0.04	93.77 ± 6.13	-0.26 ± 0.03
	"UV-visible" male	158.80 ± 6.52	-0.03 ± 0.03	168.65 ± 6.19	-0.35 ± 0.02
	"UV-absent" male	217.80 ± 7.28	0.64 ± 0.02		
C. philodice	'Alba'	25.43 ± 4.41	0.05 ± 0.03	24.55 ± 3.88	-0.06 ± 0.03
	Non-'alba'	103.26 ± 6.80	0.25 ± 0.02	61.79 ± 7.39	-0.33 ± 0.04
	Male	146.11 ± 8.82	0.28 ± 0.02	152.41 ± 7.91	-0.34 ± 0.03
P. rapae	Female	60.31 ± 8.15	0.01 ± 0.05	54.48 ± 7.03	0.10 ± 0.03

	Male	134.05 ± 6.60	-0.18 ± 0.02	99.96 ± 6.61	0.19 ± 0.02

When the data are considered in absolute terms, *C. eurytheme* 'alba' females were estimated to be discriminable from all other groups in forewing and hindwing chromatic contrast and hindwing luminance contrast (Table 2). However, for forewing luminance contrast, *P. rapae* females and *C. eurytheme* males in the 'UV-visible' orientation were not discriminable from *C. eurytheme* 'alba' females (i.e., 95% confidence interval estimates for these comparisons overlapped with zero, see Table 2, Fig. 8C). When these contrasts are considered in relative terms, some groups were more easily discriminable from each other than others (i.e. pairwise comparisons of some groups exhibited consistently higher ΔS^t and/or *L* values). *C. eurytheme* 'alba' females were least distinguishable from *C. philodice* 'alba' females for chromatic contrast of forewing and hindwing coloration, and luminance contrast for hindwing coloration (Table 2, Fig. 8). The next least distinguishable group from *C. eurytheme* 'albas' was *P. rapae* females, with intermediate chromatic contrast estimates for forewing and hindwing coloration, as well as low luminance contrast estimates for hindwing coloration (Table 2, Fig. 8). In addition, the forewing coloration of *C. eurytheme* 'alba' females exhibited the lowest luminance contrast with *P. rapae* females, *C. eurytheme* male in the "UV-visible" orientation, and *C. philodice* 'alba' females (Table 2, Fig. 8C).

3.4 DISCUSSION

We find that when viewed through the male visual system, 'alba' females of both *Colias* species should appear very bright, but not very colorful compared to a typical foliar background (Fig. 7). ‘Alba’ females have significantly higher luminance values than the background foliage, suggesting that it may be easy for a male to spot an ‘alba’ female in the field using luminance contrast. However, chromatic contrast between the ‘alba’ females and background foliage was lower than for non-‘alba’ females, which may impact male ability to use color cues to locate ‘alba’ females in the field. These patterns were consistent for ‘alba’ females of both *Colias* species. Thus, we find mixed evidence for our hypothesis that differences in mating rates between ‘alba’ and non-‘alba’ females may be driven in part by challenges males face in visually detecting either form against background foliage. If anything, we find that males should be able to readily see both female forms under typical field conditions. However, little is known about whether males prioritize chromatic versus achromatic cues during mate searching. For example, if males favor chromatic cues over luminance differences when seeking mates, this may disadvantage ‘alba’ females. More work is needed to disentangle these possibilities.

When the wing colors of different morphs/species are compared directly to each other rather than to the background, we find that all estimated chromatic contrasts fall above the 2.3 threshold typically cited as a reasonable discrimination threshold (Fig. 8, Giurfa *et al.* 1997). This suggests that males should be able to discriminate between all focal groups based on chromatic contrast, at least if males encounter these butterflies next to each other or sequentially within a short time span. Males in high population

densities may often encounter females at sufficiently high rates that a discriminability threshold of 2.3 or similar could act as a reasonable minimum. In such situations, we can tentatively conclude that males should be able to reliably discriminate between ‘alba’ females and other similarly colored co-occurring species. However, for intermediate or low population densities, males should experience females at greater distances in space or time. In such situations, a discrimination threshold of 2.3 may overestimate male capacity to reliably discriminate between female wing colors. Therefore, in field scenarios where males encounter conspecific females and heterospecific individuals at greater intervals in space or time, considering the relative chromatic contrast scores may be more informative.

When our chromatic contrast estimates are interpreted in relative terms, it becomes clear that some groups may be easier for males to discriminate than others. The ‘alba’ females of the both *Colias* species had the lowest chromatic contrast scores when compared to each other and thus should appear the most similar to the male visual system. Conversely, the chromatic contrasts between the non-‘alba’ females of the two *Colias* species were substantially larger than the chromatic contrasts between the ‘alba’ females (Fig. 8A,B, Fig S1). This indicates that males should be able to more readily discriminate between conspecific and heterospecific non-‘alba’ females than ‘alba’ females. As a result, males are likely to be able to visually identify conspecific non-‘alba’ females with greater certainty from heterospecific females when searching for mates, providing preliminary support for our hypothesis that mate-searching male *Colias* may have difficulties identifying conspecific from heterospecific ‘alba’ females using chromatic cues.

Similarly, we find low chromatic contrast scores when comparing the wing coloration of *Colias* ‘alba’ females to *P. rapae* females. However, chromatic contrast scores between ‘alba’ females and *P. rapae* males are of similar magnitude to chromatic differences between ‘alba’ females and non-‘alba’ females. Thus, our data support the hypothesis that mate-searching males may find it difficult to visually discriminate ‘alba’ females from co-flying white *Pieris* butterflies, but that this difficulty does not generalize to both sexes in *P. rapae*. Rather, *P. rapae* females are likely to pose the most significant source of uncertainty for mate-searching *Colias* males attempting to decide whether a white butterfly is reproductively profitable or not.

Results from our analysis of luminance contrasts between different butterfly color phenotypes tell a similar story. We find that ‘alba’ females exhibit the lowest luminance contrast scores with heterospecific ‘alba’ females and *P. rapae* females (Fig. 8C,D). Interestingly, we also find that for dorsal forewing coloration, *C. eurytheme* males in the “UV-visible” orientation exhibit similar luminance to ‘alba’ females, although these color phenotypes differ dramatically in chromatic contrast (Fig. 8A). Thus, males should experience additional difficulties in discriminating conspecific ‘albas’ from heterospecific ‘alba’ and *Pieris* females using luminance contrast, providing further support for our hypotheses that males may experience challenges visually discriminating between conspecific ‘alba’ females and heterospecific white butterflies. These difficulties may be important for understanding the reproductive interactions of non-‘alba’ versus ‘alba’ females.

We note that our analysis may actually provide inflated estimates of the potential discriminability of different female forms in these species, not only because of expected

reductions in discriminability between stimuli experienced at distances in space and time, but also because we have focused our measurements on freshly-eclosed individuals. Butterflies experience age-related fading of wing coloration due to the gradual loss of the wing scales that produce their coloration (Kemp 2006). Thus, age-related changes in coloration in any of our focal butterfly species may result in phenotypes that are even more difficult for mate-searching males to tell apart, either due to fading-related convergence of color phenotypes and/or increases in the phenotypic variability within each morph type leading to greater uncertainty of morph identity. Future work should explore the extent to which these age-related color dynamics influence mate attraction and male visual discrimination.

Taken together, our results provide mixed support for the idea that 'alba' and non-'alba' forms exhibit dramatic differences in visual salience when viewed by males against typical foliage backdrops. However, we do find consistent evidence that males may experience difficulties when seeking to discriminate conspecific 'albas' from heterospecific 'alba' females and co-flying *P. rapae* females using visual cues. In the field, mate-searching males face a series of tradeoffs, including speed-accuracy tradeoffs for visual discrimination (Wickelgren 1977) and decision-making (Chittka, Skorupski & Raine 2009). The reduced discrimination scores of visual comparisons between 'alba' females and other co-flying heterospecific females may thus lead to mistakes in mate choice, resulting in loss of flight time and mating resources. If such mistakes impose regular fitness costs on males, this could favor the evolution of a mating bias against 'alba' females observed by some researchers (Graham *et al.* 1980; Watt 1995). Such a bias would represent a cost to the 'alba' morph and explain why

both ‘alba’ and yellow morphs coexist in such a variety of populations despite the potential fecundity advantage enjoyed by ‘alba’ females due to lower pigment-based resource allocation (Graham *et al.* 1980). Although we did not explicitly test for the presence of a male mating bias, our work provides preliminary support for the idea that challenges to visual mate recognition may play an important role in how males interact with the female-limited color polymorphism in *Colias*.

This study is among the first to explicitly ask whether visual system limitations may influence male mate recognition in a color polymorphic species. However, we expect that sensory limitations may be of widespread importance to the evolution of traits involved in mate-attraction and mate identification, especially those exhibiting polymorphisms. For example, many species of *Heliconius* butterflies have polymorphic females that mimic different toxic species to avoid predation. This polymorphism poses a problem for visually-oriented males who must discriminate multiple female forms from multiple heterospecific model species (Kronforst *et al.* 2006). In *Heliconius cydno alithea*, this challenge appears to have been “solved” by the evolution of polymorphic mate preferences, although the visual system mechanisms underlying these assortative mating preferences are not known (Kronforst *et al.* 2006; Chamberlain *et al.* 2009).

In conclusion, our study provides a crucial first step in understanding how the male visual system might affect how males visually identify and discriminate different female color morphs in *C. eurytheme* and *C. philodice.* Our results suggest that males may, in some scenarios, experience difficulties visually discriminating between conspecific ‘alba’ females, heterospecific ‘alba’ females, and co-flying white pierids. Such visual system constraints could lead to costly mistakes by mate-searching *Colias*

males, including time lost courting heterospecifics and even higher costs associated with heterospecific mating. Thus, visual system limitations may have selected for male mating biases against ‘alba’ females, which, when counteracted by the higher fecundity of ‘alba’ females, may help to explain the evolutionary persistence of both ‘alba’ and non-‘alba’ female morphs in many species of *Colias* butterflies. We suggest that greater attention to the role of sensory perception and sensory limitations in the maintenance of polymorphic mate attraction traits is likely to be a profitable area for future work.

3.5 ACKNOWLEDGEMENTS

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4.0 BIASED, NON-PLASTIC MATE CHOICE IN THE POLYMORPHIC BUTTERFLY, *COLIAS PHILODICE*

4.1 INTRODUCTION

Polymorphisms are often used as model systems to study important ecological and evolutionary processes. They are ideal for studying larger evolutionary questions because they represent discrete intraspecific diversity, are numerous throughout varied taxa, and are easy to study because they typically have simple genetic bases and are easily observed (Svensson *et al.* 2007; Forsman *et al.* 2008). Polymorphisms are useful model systems to explore how evolutionary mechanisms maintain diversity (Ford 1945; Forsman *et al.* 2008). The most widely accepted mechanisms explaining polymorphism maintenance are either countervailing selection, where multiple different selective pressures counteract and balance each other out, or negative frequency dependent selection, where a morph's fitness decreases when its frequency rises above an equilibrium point, preventing any morph from reaching fixation. While there are many examples of negative frequency dependent selection in the literature, there are relatively few well supported instances of countervailing selection maintaining a polymorphism over evolutionary time (Roulin & Bize 2007).

Instances where polymorphism is limited to just one sex are particularly intriguing. Because of their restriction to one sex, researchers often focus on the influence of sexual selection sexual selection via mate preferences (Van Gossum,

Sherratt & Cordero-Rivera 2008). This is because mate preferences can either be involved as part of countervailing selection or can in and of themselves generate frequency dependent selection sufficient to maintain a polymorphism, e.g. damselflies (Bots *et al.* 2015). The former is not expected to involve learning, whereas the latter requires it (see further discussion below). Thus, understanding whether mates exhibit preferences for particular morphs, and whether these preferences are fixed or modified via learning, are critical pieces of information to better understand whether mate preferences may be involved in the maintenance of a sex-limited polymorphism.

There are many examples that support the existence of mate choice learning for the most common morph (van Gossum *et al.* 2001). This type of learning generates positive frequency dependence in mate choice, but may result in negative frequency dependent selection on the morphs as a result of the costs of excessive mate harassment (Svensson *et al.* 2005). There is also some evidence for neophilia, or mate choice for less common morphs (Eakley & Houde 2004). This can also be considered a form of non-associative learning, because it requires learning what the most common morph is, and then selecting something that differs from that average phenotype. In this case, the mate preferences is negatively frequency dependent and will likely result in negative frequency dependent selection on the morphs. An alternative possibility is that mates have preferences that are fixed. There are far fewer examples of fixed preferences involving mate polymorphisms in the literature, although it remains unclear whether such fixed preferences are abundant in the natural world. Fixed preferences would require a countervailing source of selection to maintain the less preferred morph in the population over time.

Here we seek to determine how a sex-limited polymorphism is being maintained in Coliadinae butterflies. Approximately half of the 203 extant species in the lepidopteran sub-family Coliadinae are characterized by a female-limited color polymorphism termed the ‘alba’ polymorphism where some females are white (termed the ‘alba’ morph) and others are yellow, orange, or red depending on the species (termed the non-‘alba’ morph). Coliadinae species are widespread, found in a broad range of environments on every continent except Antarctica (Braby 2000; Grieshuber *et al.* 2012; Limeri & Morehouse 2016). Despite the prevalence of this polymorphism, the factors that maintain both ‘alba’ and non-‘alba’ females morphs over time are not fully understood (Nielsen & Watt 2000).

To date, the operating hypothesis has been that the ‘alba’ polymorphism is maintained by countervailing selection where a male mating bias for non-‘alba’ females is counteracted by a developmental advantage that ‘alba’ females gain by redirecting important nutrients from pterin pigment production to developmental processes (Watt 1973; Graham *et al.* 1980; Nielsen & Watt 1998). Recent modeling of the male visual system indicates that males may have difficulty visually discriminating between conspecific ‘alba’ females and heterospecific white butterflies, such as pierids or ‘alba’ females of other Coliadinae species (Limeri & Morehouse 2014). This challenge to visual discrimination may lead males to prefer to mate with the more distinctive non-‘alba’ female morph. Researchers have observed such a mate preference in some populations (Graham *et al.* 1980; Nielsen & Watt 1998, 2000). Further, there is some evidence for population-level variation in mate preferences that may be related to the frequency of white butterflies in the community (Nielsen & Watt 2000). A comparison of

two different species in the genus *Colias* revealed that the males of a species that co-occurs with a high density of heterospecific white pierids that resemble ‘alba’ females have a stronger preference for non-‘alba’ females than males of a species with little contact with white pierids (Nielsen & Watt 2000). While these results suggest that male *Colias* butterflies may adjust their mate preferences in response to changing population demographics, it remains unclear whether this is due to genetic changes to fixed preferences or more rapid phenotypic plasticity of male mate preferences through mate choice learning (i.e., frequency dependent mating preferences as described earlier). However, researchers have yet to specifically test for mate preference learning in the Coliadinae. Therefore, we aim to verify the hypothesized mate preference for non-‘alba’ females and to specifically test for whether this preference can be modified via learning.

In this study, we tested whether males of the Clouded Sulphur butterfly, *Colias philodice*, exhibit mating preferences with regard to female color morph. This species is amenable to rearing in a laboratory setting and has been the focal species for many foundational investigations of this female-limited polymorphism (e.g., (Remington 1954; Taylor, Grula & Hayes 1981; Marshall 1982; Limeri & Morehouse 2014). We further asked whether males adjust their mate preferences in response to changes in the morph frequencies they experience in their local population. We tested this both correlatively using variation in morph frequency and male preference in natural populations and manipulatively using experimental populations of captive butterflies.

4.2 METHODS

4.2.1 Field observations

The clouded sulfur butterfly, *C. philodice*, is widespread and abundant throughout North America. They inhabit open fields and disturbed habitats and utilize a variety of legumes as larval hosts, most commonly alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), or vetch (*Vicia faba*) (Scott 1997). Examinations of museum collections indicates that polymorphism frequencies vary throughout their range with 'alba' females being more abundant in northern populations and non-'alba' females being more abundant in southern populations (Hovanitz 1944). In southwestern Pennsylvania, polymorphism frequencies are highly variable across space and time, ranging between 20% and 100% 'alba' females in local sub-populations (unpublished data).

We studied natural populations of *C. philodice* at five different agricultural sites in southwestern Pennsylvania: Cochranton (41.501602°N, -80.019445°W), Greensburg (40.287920°N, -79.442209°W), Rochester (40.733944°N, -80.161031°W), and Aliquippa (40.567814°N, -80.350139°W). These animals can complete their life cycle on cultivated alfalfa (*Medicago sativa*). Thus, agricultural alfalfa fields harbor large, persistent populations of these butterflies throughout the growing season. We collected demographic and male preference data once a month every month during the flight season, May through September of 2015 and April through September of 2016.

We first measured the demographic composition of the adult butterflies at these sites using a transect method. Members of the research team walked linear transects (~325 m long) through alfalfa fields at a steady pace for five minutes. During these transects, we counted the number of *Pieris rapae*, *Colias philodice*, and *Colias eurytheme* individuals within a 5m radius in front of or to the sides of the transect path. When the identity of the butterfly was uncertain, it was captured in net to confirm identity. 'Alba' females of both species were combined into one category because they cannot be readily distinguished, even once in hand. The transect was paused while an unidentified butterfly was being captured and identified in net, or while clouds blocked the sun, the latter because the butterflies become much less active without direct sunlight. Four transects were conducted at each field site during each monthly visit.

We then quantified temporal variation in wild male mate preferences by observing male approaches to an array of artificial, mechanically moving female models. The female models consisted of a black plastic body attached to wings printed and colored on paper. The butterfly model was attached via a thin metal wire to a solar-powered spinning stand, which created fluttering movement. The models were created by modifying a "Solar Powered Flying Wobble Fluttering Butterfly" (YR.Seasons via Amazon.com, Inc., Seattle, WA).

Model 'wings' were created by printing the black wing pattern elements on heavyweight acid-free paper (up & up brand, Target, Minneapolis, MN), followed by adding in morph color using art markers (Copic, Tokyo, Japan). The non-'alba' phenotype was produced using the Copic marker color "Y02" and the 'alba' phenotype was produced using the marker color "T2." We selected these markers by comparing

the reflectance spectra of the models to the reflectance spectra of real ‘alba’ and non-‘alba’ female wings. Measurements were taken following previously described methods (Morehouse & Rutowski 2010; Limeri & Morehouse 2014) using a spectrometer (USB 2000+, Ocean Optics, Inc., Dunedin, FL). Briefly, wing/model surfaces were illuminated using a deuterium/tungsten/halogen light source (DH2000-BAL, Ocean Optics, Inc.), with its collimated beam oriented normal to the wing/model surface. Light reflected from the wing surface was then sampled at a 45° azimuth towards the proximal wing margin along the distal-proximal axis of the wing using a collimated fiber optic collector connected to the spectrophotometer. Measurements from 300-700 nm were taken relative to a magnesium oxide white standard using SpectraSuite software (Ocean Optics, integration time: 200ms, number of spectra averaged: 25, boxcar width: 8). We first used visual inspection of resulting spectra to evaluate a wide range of commercially available markers. We found that Y02 and T2 were the marker colors that allowed us to best approximate the spectral properties of the corresponding butterfly phenotypes (Fig. 9). To more formally assess how accurately the model colors resembled the phenotypes of real *C. philodice* wings, we compared these spectra using our previously published model of *Colias* color vision (Limeri & Morehouse 2014). We used a Weber fraction of 0.05 following a common historical precedent in visual models (Endler & Mielke 2005). However in the past we have also used a Weber fraction of 0.01 which is more conservative. Therefore, we ran a set of contrasts using both values to ensure that the difference did not qualitatively change the results and we found that both Weber fractions yielded qualitatively similar results. Model spectra were compared to the average spectra of the forewings of eight lab-reared, freshly eclosed ‘alba’ and non-

‘alba’ females. The ‘alba’ model differed from natural ‘alba’ females by eight standard deviations of photoreceptor noise on average in chromatic contrast and -0.1 standard deviations of photoreceptor noise in luminance contrast. The non-‘alba’ model differed from natural non-‘alba’ females by 10 standard deviations of photoreceptor noise in chromatic contrast and -0.2 standard deviations of photoreceptor noise in luminance contrast. These suggest that while our butterfly models were not exact matches, they were very close approximations.

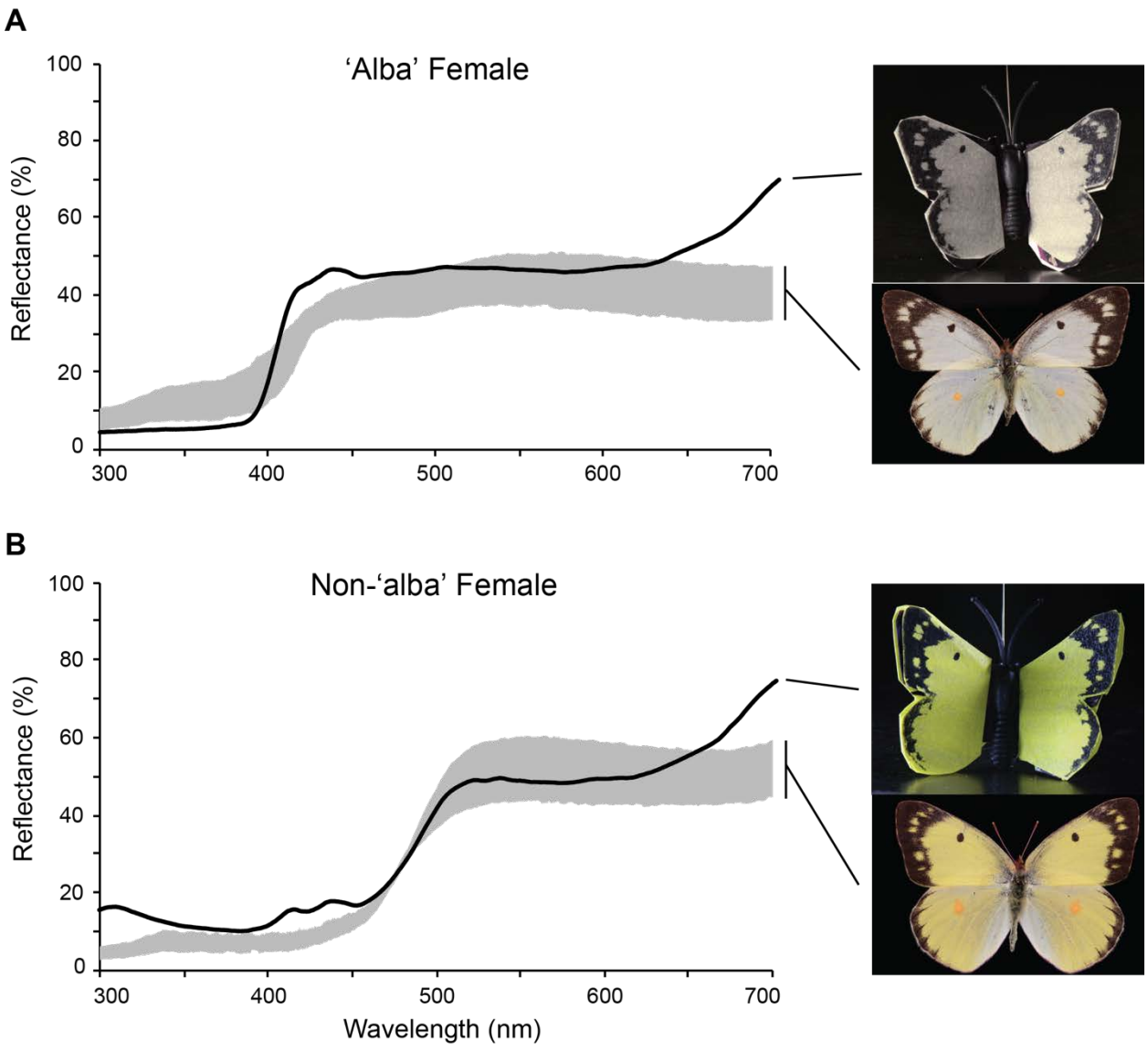

Figure 9. Comparison of spectra of models and real butterflies

Spectral reflectance measurements of real 'alba' (A) and non-'alba' (B) females compared to female models used in field observations of male mate preferences. The shaded section represents the range of values in a sample of eight freshly eclosed lab-reared females and the solid lines represent the measured model reflectance spectra.

Male mate preferences were quantified by counting the number of times each type of model was approached by males during a 90 minute observation period during

each monthly visit (observations were paused during cloudy conditions). It is possible that a male may have visited the array more than once and have been counted as separate, independent visits, which would introduce pseudo-replication issues. However, this is unlikely because males move quickly over long distances and migration among populations is high. Although we consider it highly unlikely for these reasons, it is possible a male may have been counted more than once.

Six models consisting of two ‘alba’ models, two *C. philodice* non-‘alba’ models, and two *C. eurytheme* non-‘alba’ models were placed in a rectangular array with models spaced 1.5m apart. Model positions in the array were rotated every 15 min during the observation period such that every model spent the same amount of time in each position to eliminate directional biases. *C. eurytheme* non-‘alba’ female models were included as a positive control to ensure that males were not approaching models randomly. *C. philodice* males approached *C. philodice* non-‘alba’ models far more frequently than they approached *C. eurytheme* non-‘alba’ models. In all analyses presented here, any approaches to *C. eurytheme* female models are excluded. The observer sat 10m away from the array to minimize interference with male behavior while being able to readily record the identity and approach behavior of mate-searching males. We only recorded the first approach of *C. philodice* males.

4.2.2 Behavioral experiment

For behavioral experiments, we reared butterflies in lab colonies initiated by capturing wild *C. philodice* females from the four field sites in which field observations

were conducted (see above) and bringing them into lab to lay eggs. The offspring were then raised on fresh alfalfa (*Medicago sativa*) harvested from the same agricultural fields. The butterflies used in the first seven trials were reared in a room that maintained a steady 22°C temperature, but with relative humidity that fluctuated with the weather conditions. These caterpillars were exposed to natural sunlight and day length. Butterflies for the remaining 41 trials were reared in climate chambers that maintained a constant temperature (24°C) and humidity (55% RH), with photoperiod cycle mimicking mid-summer conditions (16:8, light:dark). Caterpillars were raised in small groups of approximately 10 caterpillars per container until pupation, at which point they were isolated into individual containers. After emergence, butterflies were kept in these individual containers until they were used in experiments, no more than 3 days later.

We tested whether these lab-reared males express an innate preference between morphs and whether this preference is flexible by measuring male preferences after experience with different population compositions. Cohorts of five males were exposed to one of three experimental population treatments: mostly ‘alba’ treatment (nine ‘alba’ females and one non-‘alba’ female), equal ratio treatment (five ‘alba’ and five non-‘alba’ females), or mostly non-‘alba’ treatment (one ‘alba’ and nine non-‘alba’ females). These experimental populations, composed of five males along with ten females, were placed in 2m x 1.5m x 1.2m mesh enclosures. Enclosures were constructed from PVC piping and white polyester fabric (Bone signature voile, Item # 8139909 from Jo-Ann Fabrics, Hudson, OH, USA), with entrances secured with Velcro. We selected this particular fabric because, of the fabrics available, this fabric resulted in the greatest light transmission while leaving the spectral properties of incident irradiance

relatively unaltered. We formally evaluated these light transmission properties using a field portable, calibrated spectrophotometer (Jaz, Ocean Optics, Inc., Dunedin, FL), with the following settings: integration time: 26ms, spectra averaged: 30, and boxcar smoothing: 5 (Fig. S2). Absolute irradiance calibrations were done with a standard light source (LS1-CAL Ocean Optics, Inc., Dunedin, FL).

Experimental populations were placed in enclosures during the morning of the first day of their testing. During June and July, this occurred at 9:00, and in August and September, this occurred at 10:00 due to the chillier weather in the early morning. The butterflies were allowed to freely interact for three hours. The exposure period occurred during the morning because these butterflies are most active during the morning and most first-matings occur in the morning (Forsberg & Wiklund 1989). These butterflies have a short lifespan of these butterflies in natural environments, living only 2-7 days (Watt *et al.* 1977; Kingsolver 1983). Therefore, one morning is a significant portion of their overall lifespan.

Enclosures were checked every 20 minutes for matings, a frequency that falls well within the typical average mating time of 45 minutes (Rutowski & Gilchrist 1986). Matings were allowed to proceed uninterrupted and the wings of mated females were marked with a small line to avoid using non-virgin females in later parts of the experiment. After three hours, the females were removed, and the males were left in the enclosures overnight. However, toward the end of the experiment, 12 cohorts of males (out of a total of 46 cohorts of males) were returned to isolated compartments and kept inside overnight to improve survivorship and prevent escape. These latter 12 cohorts were approximately evenly distributed amongst the treatment types, so this

experimental difference is unlikely to have systematically biased the outcomes of any given treatment.

The following morning, mate preferences were tested in two phases that occurred in haphazard order: the unaltered female preference test and the altered phenotype female preference test. The unaltered female preference test was conducted by placing a single male in a small mesh enclosure with two ‘alba’ and two non-‘alba’ naïve, freshly-elcosed, virgin females. Male behavior towards females was observed for thirty minutes. Male behavior was recorded as “approach” or “mating attempt.” An “approach” was scored each time a male moved within one body length of a female while facing her but did not attempt to mate. A “mating attempt” was scored every time a male climbed on top of a female, attempted to land on a female, or bent his abdomen towards a female’s, even if he failed to connect. When a male did successfully connect abdomens with a female, we would physically separate the pair to prevent copulation and release the male and female back into the mesh enclosure. At the end of the thirty-minute period, one male was removed and the next male in the cohort was added, such that all five males in the same cohort were tested using the same four females. The order in which males were tested was haphazard and trial order was noted to evaluate whether the repeated use of the same focal females influenced male behaviors. The non-independence introduced by this method was taken into consideration during statistical analyses (see below).

In the altered phenotype female preference test, mate preferences were tested using females whose morph appearance had been altered using bleaching and artificial coloring. This test allowed us to isolate the effects of female wing color from other

potentially correlated traits, such as female behavior or pheromones. To alter female color phenotypes, we removed pigments from the female wings following previously established methods (Morehouse & Rutowski 2010) and added color using Copic brand art markers (Y02 and T2, see above). Females were held with forceps at the base of their wings and were quickly dipped in 100% ethanol to surmount the natural hydrophobicity of butterfly wings. Wings were then soaked in a dilute bleach solution (1% hypochlorite) for 30 seconds to remove pterin pigments from the wings while keeping the structure and dark melanin pigments intact. Wings were then dipped in ethanol again to remove the bleach and allowed to air dry. Finally, the wings were re-colored with Copic Sketch brand art markers. As with the paper models, the non-‘alba’ phenotype was produced using color “Y02” and the ‘alba’ phenotype was produced using the marker color “T2.” Each female was bleached and re-colored as either their original phenotype (control) or the opposite phenotype (phenotype-swapped), creating four total possible combinations (two control females and two phenotype-swapped females). These combinations allowed us to test whether male preferences are based on wing color or other traits, such as behavior, that are correlated with natural morph phenotype. In the former case, males would prefer white or yellow phenotype-altered females regardless of their original identity. Conversely, if male preferences are based on a trait other than wing color that is correlated with natural morph phenotype, male preferences would be based on the original identity of the female, regardless of the altered color of the female’s wings.

Spectral measurements were collected from ten of each type of altered phenotype females and compared to the spectra of unaltered female wings (the same

eight ‘alba’ and eight non-‘alba’ used for comparison to butterfly models above; Fig. 10). As with the model contrasts, we used a receptor-noise-limited model of *C. philodice* color vision to assess correspondence between the spectra of altered and unaltered females (Table S2). We analyzed these contrasts using a Weber value of 0.01 as used in the original model and 0.05 which is consistent with other visual models in the literature (Endler & Mielke 2005). Contrasts with both these values show the same pattern where phenotype-altered females much more closely resemble the phenotype they are intended to mimic than the opposite phenotype (Table S2).

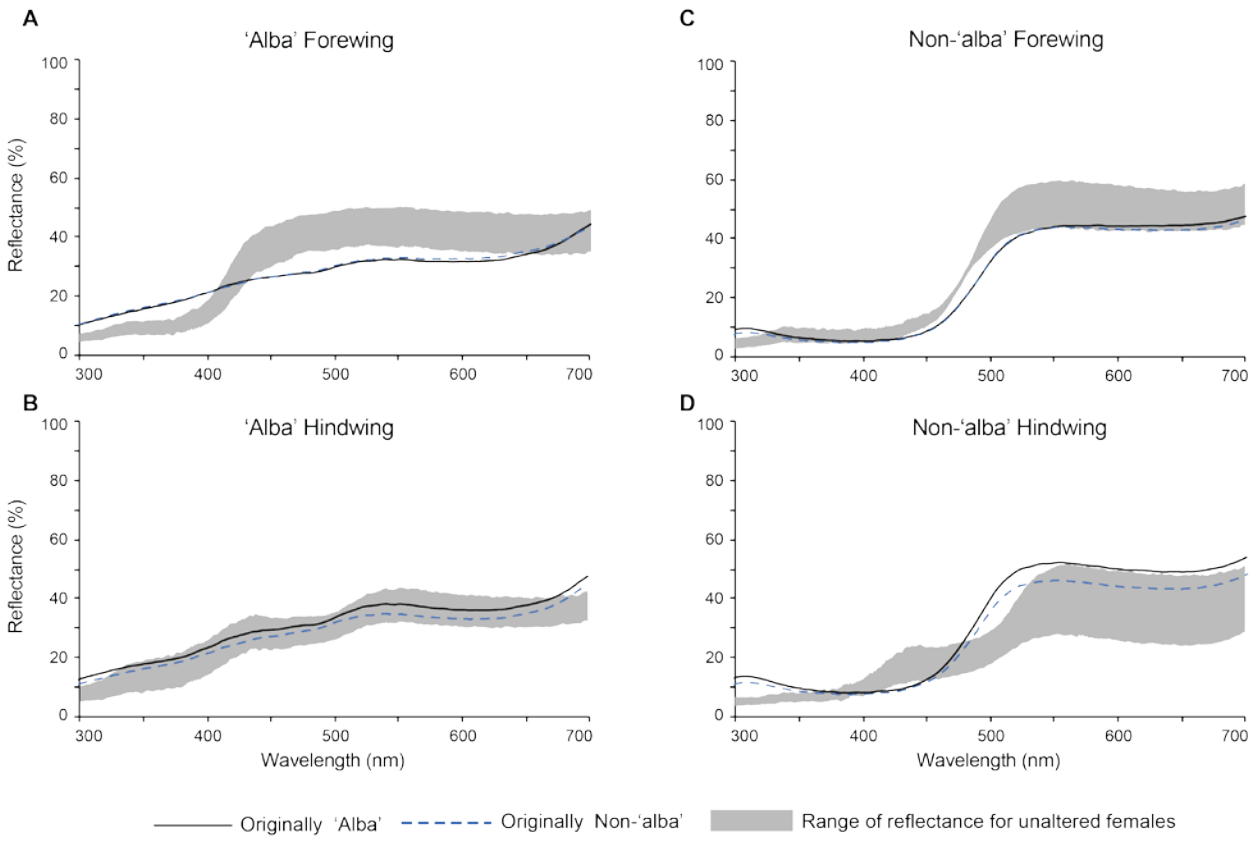

Figure 10. Comparison of spectra of models and real butterflies

Spectral reflectance of phenotype-altered females to natural ‘alba’ (A and B) and natural non-‘alba’ (C and D) female wings. The females that were altered to resemble ‘alba’ females (originally ‘alba’ females

bleached and recolored as ‘alba’ and originally non-‘alba’ female bleached and re-colored as ‘alba’) are compared to unaltered ‘alba’ females (A and B). The females that were altered to resemble non-‘alba’ females (originally ‘alba’ females bleached and recolored as non-‘alba’ and originally non-‘alba’ female bleached and re-colored as non-‘alba’) are compared to unaltered non-‘alba’ females (C and D). The shaded area represents the range of natural variation in a sample of eight unaltered ‘alba’ and eight unaltered non-‘alba’ females.

We were able to achieve a reasonably good match based on visual examination of the reflectance spectra on three of the four wing surfaces (‘alba’ hindwing, non-‘alba’ forewing and hindwing). However, correspondence between the altered ‘alba’ forewing to the unaltered ‘alba forewing was poor because the reflectance spectra of the altered phenotype females fell largely outside the range of natural variation (Fig. 10). Males in our preference trials largely interacted with females whose wings were closed over their backs, and thus are likely to have assessed females based predominantly on ventral coloration rather than dorsal coloration. However, this poor correspondence in dorsal coloration between altered and unaltered females requires cautious interpretations of the results of this assay.

Each male was tested with four altered phenotype females, two phenotype-swapped females and two control females. Phenotype-altered females were less active than unaltered females, but were observed to fly, feed, attract male attention, and mate. Similar to the unaltered female test, each male was placed in a small mesh enclosure with four altered phenotype females and observed for thirty minutes. At the end of the thirty minutes, that male was removed and replaced with another male from the same

cohort, such that each cohort of five males was tested with the same set of altered phenotype females. The order in which males were tested was haphazard.

In total, 48 cohorts of males were tested (14 in the mostly ‘alba’ treatment, 15 in the equal ratio treatment, and 19 in the mostly non-‘alba’ treatment). Thirty-four of these cohorts were tested in 2015 and 14 were tested in 2016. Seven of the cohorts in 2015 were performed at the Pymatuning Laboratory of Ecology in Linesville, PA (41.569053°N, -80.466395°W). Based on availability of location, twelve of the cohorts tested in 2016 were conducted on the roof of the University of Pittsburgh’s Biological Sciences building in Pittsburgh, PA (40.445568°N, -79.953458°W) and were provided nectaring plants in lieu of a grassy turf. The remaining 22 cohorts were tested on a grassy lawn next to the Biological Sciences building. The treatments performed in each of these locations was haphazard and approximately equal, so this should not bias results.

4.2.3 Field data analysis

All data were analyzed using R v3.3.1 (R Development Core Team 2011). We first tested whether male preferences for ‘alba’ versus non-‘alba’ models were a function of the proportion of ‘alba’ females in a given population using Generalized Estimating Equations (GEEs). GEEs are a semi-parametric regression technique that estimates the parameters of a generalized linear model and can handle many types of unmeasured dependence between outcomes (Liang & Zeger 1986; Prentice & Zhao 1991). GEEs are robust to non-independence between outcomes because they estimate the average

response over the population ("population-averaged" effects) rather than parameters for a model that predicts the outcome of each individual. We chose to analyze our field observations using GEEs because these data are both spatially and temporally non-independent, as a result of repeated measures (temporal non-independence) of populations at sites that are not equally spaced apart (spatial non-independence). A major strength of GEEs is the ability to account for temporal non-independence by using an autoregressive correlation structure (ar1). We used the proportion of all approaches to the models that were directed towards ‘alba’ females during one 90-minute observation session as the outcome variable in the model. There was a lot of variation in the total number of approaches to the array during an observation session due to factors such as temperature, cloudiness, and mowing cycle of the alfalfa field in which these trials took place. Time points where zero males approached the array were excluded from the model. The number of approaches towards the array ranged from 1 to 41. This high variability in sample size creates an unequal variance between time points. The predictor variable was the proportion of females in the population that were ‘alba’ females on the day the male mate preferences were observed. The model was run with site as the clustering variable and an autoregressive correlation structure (ar1). We conducted this analysis using the geeglm() function from the “geepack” library (Ulrich, Højsgaard & Yan 2006).

4.2.4 Experiment data analysis

We then analyzed data from the experiment to determine whether males express a mate preference for ‘alba’ or non-‘alba’ females, what females traits these preferences

are based on, and whether prior experience can affect these male mate preferences. First, we tested whether male preferences are altered by experience by testing male preferences for unaltered females. Then, we tested whether male preferences are based on female wing color or another trait correlated with natural female phenotype. We evaluated the former by testing male preferences between the wing color of altered phenotype females (regardless of their original identity) and the latter by testing male preferences between the original phenotypes of altered phenotype females (regardless of their new wing color). We conducted all of these tests using binomial generalized linear mixed effects models (GLMERs). Male mate preferences were estimated based on the number of times males approached and attempted to mate with females of each morph (hereafter any of these interactions is called a “choice”). For these models we included “cohort” as a random factor and choice as repeated measures within cohort. GLMERs were carried out using the glmer() function in the lme4 package and specifying type III Wald chi-square ($\chi 2$) tests via the Anova() function in the car package (Bates *et al.* 2015; Fox 2015). Statistical tests were run with all data including approaches because sample sizes without approaches were be too small to provide sufficient statistical power.

4.3 RESULTS

4.3.1 Field data results

Both wild male mate preferences and the proportion of ‘alba’ females in the communities were highly variable over both space and time (Fig. 11A). Plotting the proportion of approaches towards ‘alba’ models against the proportion of ‘alba’ females in the community shows no obvious trend (Fig. 11A). The generalized estimating equations revealed that the proportion of ‘alba’ females in the community did not influence the mating preferences of wild males in extant agricultural *Colias* populations (p = 0.10). These results indicate that wild male preferences do not change based on the community they are in.

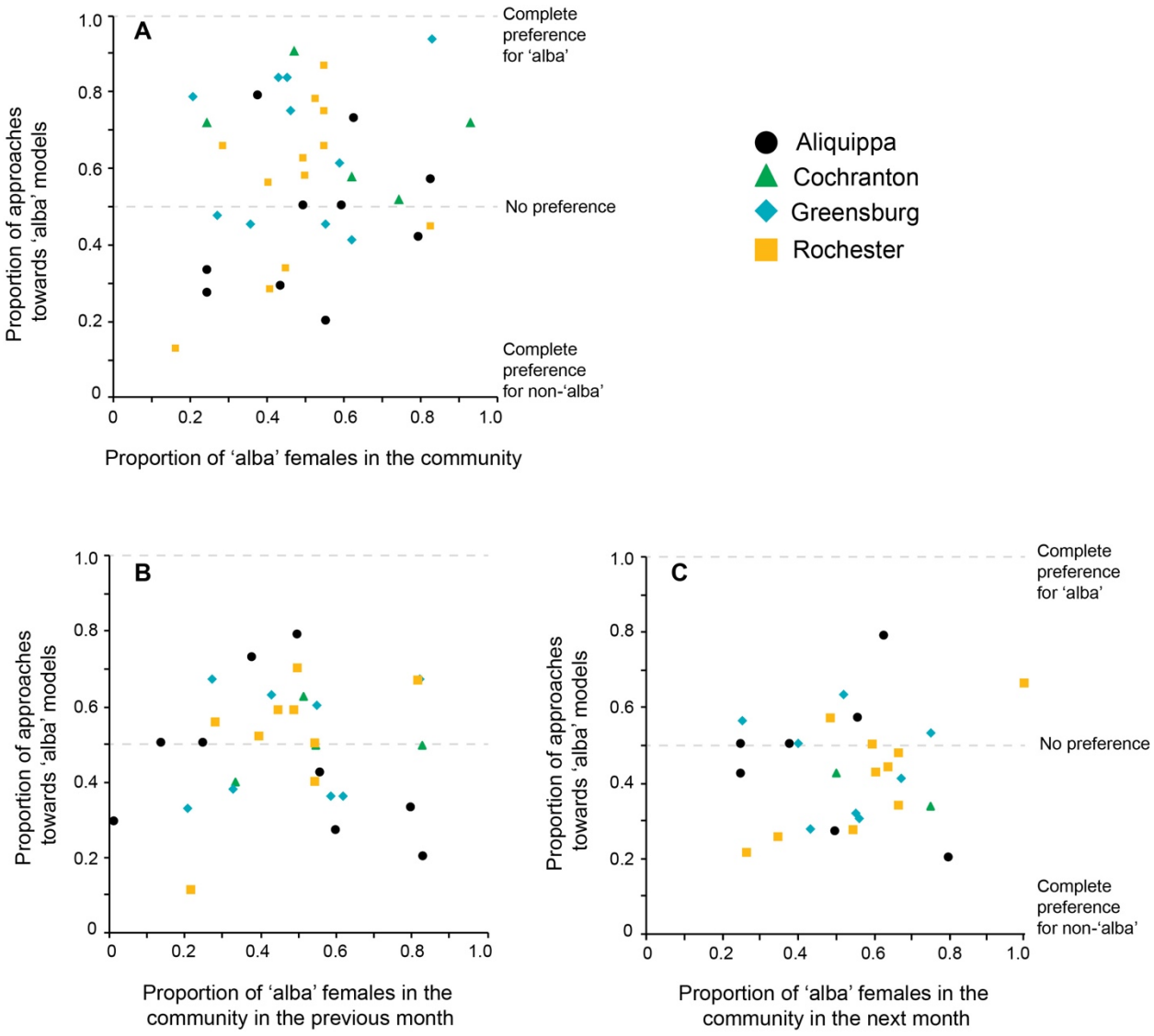

Figure 11. Wild male approaches

The proportion of wild male approaches towards 'alba' models over the proportion of 'alba' females in the community simultaneously (A) and at time lags with 'alba' proportion predicting male preferences (B) and male preferences predicting 'alba' proportion (C). Different field locations are represented by different symbols and colors.

We also explored whether male preferences might be influenced with a time delay (i.e., that males slowly learn from the extant composition, and apply these learned

preferences to subsequent populations) by testing whether male preferences are influenced by the proportion of ‘alba’ females in the prior month. The generalized estimating equations revealed that male preferences were not affected by the proportion of ‘alba’ females in the community in the prior month (Fig 11B, $p = 0.55$).

We then investigated whether male preferences influence the proportion of ‘alba’ females in the next generation (i.e. that if male preference drives female morph fitness, male preferences may affect future morph ratios). Generalized estimating equations revealed that male mate preferences did not affect the proportion of ‘alba’ females in the next month (Fig 11C, $p = 0.42$).

4.3.2 Experimental results

First we asked whether males exhibit a preference for either morph. Preferences were estimated based on the frequency of approaches and mating attempts males made towards each female morph. Approaches were by far the most common interaction, but may not be an accurate estimate of male preference because the males and females were in a small container during the mate preference testing phase and some approaches may have been coincidental rather than a sign of mating interest. Therefore, we considered preference based on all interactions, and then subsequently, excluding approaches. When both approaches and mating attempts are included, there is no clear evidence for a male mate preference. Males in the equal ratio treatment were 1.2 times more likely to approach or attempt to mate with non-‘alba’ as ‘alba’ females. However, when approaches were excluded, a stronger preference for non-‘alba’

females emerges. Males in the equal ratio treatment were 2.5 times as likely to attempt to mate with non-‘alba’ females as ‘alba’ females.

Next we determined if male preferences are influenced by their experiences with different community compositions. If male preferences were altered by the community, we would expect to find consistent differences in preference between the three different community experience treatments. Here we also considered preferences using both approaches and attempted matings (Fig. 12A) and excluding approaches (Fig. 12B). Whether approaches are included or excluded, there does not appear to be a clear effect of prior community experience because preferences do not consistently vary among treatment groups in either case (Fig 12).

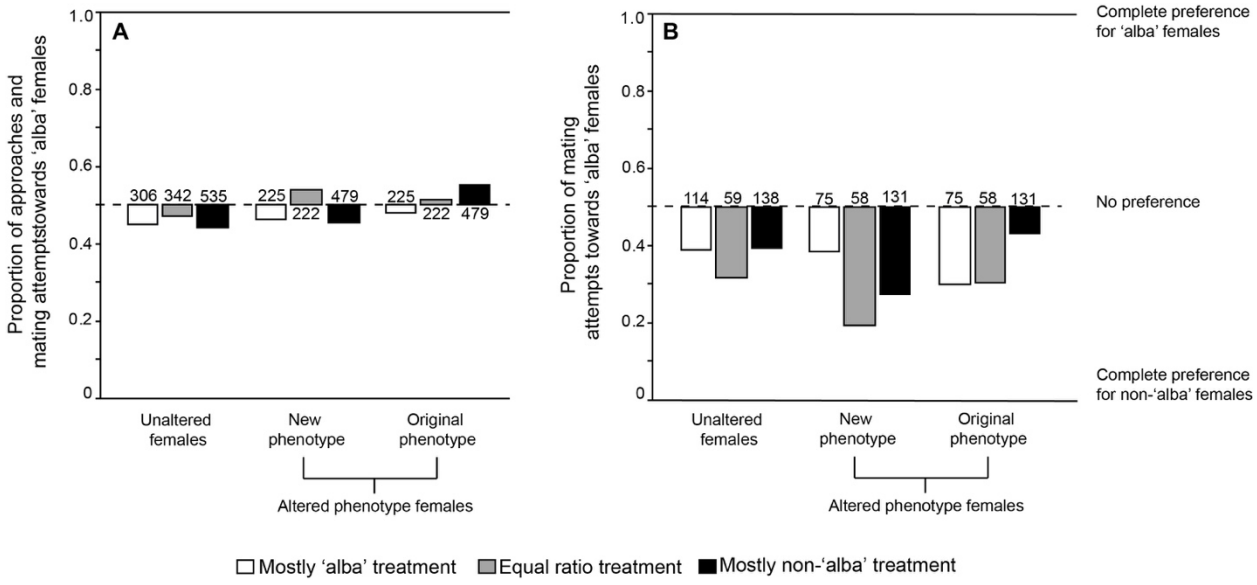

Figure 12. Male mate preferences across treatments

Male mate preferences among treatments for unaltered and altered females. Male mate preferences are estimated two ways: including both the proportion of mating attempts and approaches towards ‘alba’ females (A) and only including the proportion of mating attempts towards ‘alba’ females (B). The number of interactions that each preference estimate is based on is represented by numbers at the base of the

bars. A value of 0.5 represent an equal amount of male interaction with each morph type, indicating no preference. A value higher than 0.5 represents a preference for ‘alba’ females and a value lower than 0.5 represents a preference for non-‘alba’ females.

We ran three different tests to determine whether treatment affected male preference. Our first analysis found that the ratio of female morphs a male experienced prior to testing had no effect on a male’s preference for unaltered females (Type III Wald χ^2 test for treatment × mate preference: $\chi^2 = 0.22$, df = 2, $p = 0.90$). The second two analyses were based on male choices between the altered phenotype females. We found that males in different treatments did not show any difference in preference for the new phenotype of altered phenotype females (Type III Wald χ^2 test for treatment × mate preference: $\chi^2 = 0.47$, df = 2, $p = 0.79$). Finally, males also did not show a difference in preference for the original identity of altered phenotype females (Type III Wald χ^2 test for treatment × mate preference: $\chi^2 = 0.57$, df = 2, $p = 0.75$). These results indicate that prior experience does not affect male preference on the basis of either color or other traits.

4.4 DISCUSSION

We tested whether male *C. philodice* have a preference for ‘alba’ or non-‘alba’ females and whether male mating preferences are influenced by prior experience. We find that males approach ‘alba’ and non-‘alba’ females indiscriminately but prefer to court non-‘alba’ females. This finding corroborates previous reports that males prefer to

mate with non-‘alba’ females (Nielsen & Watt 2000; Kemp & Macedonia 2006). Whether male attention is beneficial or harmful to females depends on the environment (Nielsen & Watt 2000). In high density environments, females subject to excessive male attention are likely to suffer fitness costs associated with too much male attention, such as loss of flight time and needing to perform energetically expensive “ascending flights” to reject male courtship attempts (Rutowski 1978; Rutowski & Gilchrist 1986). In this case, male attention would be disadvantageous for the preferred female morph (Nielsen & Watt 1998). Conversely, in a low density environment where females may have a difficult time finding a mate when they are ready to mate, being the favored morph is likely to be an advantage. The preferred morph would spend less time waiting for the first mating, and therefore begin laying fertilized eggs sooner after emergence. Furthermore, *C. philodice* females typically mate up to three times during their lifespan and gather important nutrients from males during a mating (Boggs & Watt 1981). Consequently, the disfavored females may also experience a delay in obtaining these important male-derived nutrients during mating (Nielsen & Watt 1998, 2000). Thus, male mate preferences impact female *C. philodice* fitness and therefore likely play a role in the maintenance of the ‘alba’ polymorphism.

Our field data and experiment data capture male preferences at different stages of the courtship process. Our field data reveals male likelihood to approach and investigate yellow or white stimuli from afar whereas our behavioral assays in the lab reveal male preferences once males and females are already in close proximity. Our field data show that male preferences for approaching female morphs do vary, but do not consistently co-vary with community composition. In contrast, our experimental data

show that males are consistently more likely to court non-‘alba’ females once in close proximity. However, consistent with our field data, these “close-range” preferences are unaltered by community composition. Thus, neither stage of courtship appears to be influenced by learning. It is unclear why preferences should differ between these two stages of courtship. One possibility is that investigating a stimulus incurs very little cost and has a potential of high payoff, so males might be best served by indiscriminately approaching females. However, courting a female is a larger investment so males are more selective about courtship decisions. Our results emphasize the importance of considering all stages of courtship when attempting to measure preferences in any system.

Our finding that male preferences do not change with morph frequency is in contrast to a number of other systems, where variable mate preferences appear to drive frequency dependent selection (Ten Cate & Vos 1999; Magurran & Ramnarine 2004; Dukas 2008; Verzijden *et al.* 2012). Examples of directional, non-plastic preferences in polymorphic species are uncommon in the literature and the impacts of the directional preferences on polymorphism maintenance are poorly understood (Roulin & Bize 2007).

There are four possible outcomes from non-plastic preferences in a polymorphic system. First, the polymorphism could be transient and directional preferences are actively driving one or more morphs to extinction. This appears to not be the general case in *Colias*; a phylogenetic study revealed that the polymorphism is likely ancestral to the entire Coliadinae sub-family (Limeri & Morehouse 2016) and that it is retained in roughly half of the extant species in this clade. However, we do note that this implies that half of the species in the Coliadinae have lost one morph, suggesting that this

polymorphism may be somewhat unstable over longer stretches of evolutionary time. It is noteworthy that there appears to be a weak bias favoring the fixation of non-‘alba’ females: of the 103 monomorphic Coliadinae species, 63 (61%) have only non-‘alba’ females and 40 (39%) have only ‘alba’ females (Limeri & Morehouse 2016). This could indicate that male mate preferences for non-‘alba’ females help drive some of these transitions. An alternative explanation is that one or more lineages which had reverted to non-‘alba’ monomorphism coincidentally resulted in more daughter species than lineages that had reverted to ‘alba’ monomorphism. Further, it’s possible that species are more likely to experience conditions that favor that non-‘alba’ morph in other ways, leading to fixation of the non-‘alba’ morph more frequently than the ‘alba’ morph.

Second, it is possible that there is individual-level variation in preferences, with different individuals exhibiting fixed preferences for different morphs, e.g. Gouldian finches prefer to mate with their own morph (Gilby, Pryke & Griffith 2009). Our experimental design was not geared towards robust estimates of individual-level variation in preference, but we don’t see any clear evidence for alternative mating preferences in male *C. philodice*. Nevertheless, it is possible that the average preference for non-‘alba’ females that we report actually obscures important underlying variation in male preferences. However, even if true, if our experimental males are representative of their source population, this would still imply a fitness advantage for non-‘alba’ females with regard to the majority male mating preference.

Third, directional preferences could maintain a polymorphism if they varied temporally or spatially. There are several accounts of varying preferences, such as seasonally varying mating preferences in the ladybird beetle *Harmonia asyridis* (Osawa

& Nishida 1992), but it remains unclear whether these varying preferences are sufficient to maintain the polymorphism (Hendrick 1986; Barton & Turelli 1989; Ellner & Hairston 1994). Our data demonstrate that mate preferences do not consistently vary temporally or spatially in *C. philodice*, at least within the spatio-temporal scale upon which our study took place. Field males were sampled repeatedly across the flight season over two years in this species, and we do not detect any evidence for regular seasonal shifts in male interest in model phenotypes.

Fourth, there could be countervailing selection where directional mate preferences are countered by some other factor. These are often described as alternative mating strategies, where one mating strategy is associated with a strong mate preference, whereas the other strategy makes up for reduced mating interest by reducing other costs (e.g. avoiding the costs of male-male conflict, for example sunfish Neff, Fu & Gross 2003). An example where different morphs adopt alternative mating strategies is the side-blotched lizard, where some morphs are territorial and others are "sneaker" males (Sinervo & Lively 1996). While alternative mating strategies are commonly hypothesized to explain polymorphism maintenance, there are very few empirical studies confirming this in a color polymorphic system (Roulin & Bize 2007). There is even less evidence of directional mating preferences being traded off against some other, non-sexually selected advantage (Roulin & Bize 2007).

There are several cases where directional mating preferences have been reported but it is not known which of the four outcomes discussed above is occurring (e.g. sockeye salmon, *Oncorhynchus nerka,* Craig and Foote 2001; and pentamorphic fish, *Poecilia parae,* Bourne et al. 2003). Here we demonstrate that male *C. philodice*

have a directional preference for the non-‘alba’ female morph that does not consistently vary temporally or spatially. While our experiment was not designed to test whether males have alternative directional mating preferences, we see no evidence supporting this. We can further rule out the hypothesis that the polymorphism is transient because phylogenetic analysis reveals that the ancestor of the Coliadinae was polymorphic and that polymorphism has been maintained in many lineages over long evolutionary periods of time (Limeri & Morehouse 2016). This leaves countervailing selection as the most likely hypothesis explaining the maintenance of the ‘alba’ polymorphism. The constant, directional selective pressure exerted by male preferences must be counteracted by some other factor in order for both morphs to be maintained in the population over time. One possibility is that the ‘alba’ polymorphism is maintained by a tradeoff between the selective pressures imposed on each morph by male mate preferences and metabolic differences between the two morphs which produce different types and quantities of nitrogen-rich pterin pigments (Watt 1973; Nielsen & Watt 2000).

An alternative explanation is that even though male preferences are detectable, they are not very strong and do not have a significant impact on female fitness. This seems unlikely given that even small impacts on morph fitness should have significant consequences over evolutionary time.

4.5 CONCLUSION

We find that male *C. philodice* have a non-flexible preference to mate with the non-‘alba’ female morph and that this preference is not influenced by the morph ratio in the population. In order for the polymorphism to be maintained, the selective forces imposed by a male mating preference for the non-‘alba’ morph must be countered by some other selective pressure.

While a mating bias towards non-‘alba’ females has been observed in the field, this is the first time a directional, non-frequency dependent preference for non-‘alba’ females has been empirically demonstrated.

4.6 ACKNOWLEDGEMENTS

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5.0 OPTIMAL MATE CHOICE IN POLYMORPHIC SYSTEMS: DISCRIMINABILITY, COMMUNITY COMPOSITION, AND MORPH-SPECIFIC CONSEQUENCES

5.1 INTRODUCTION

Decision-making is ubiquitous in the lives of all animals. Animals must often rapidly decide between multiple options, such as selecting between food types or choosing amongst multiple prospective mates (Abbott & Sherratt 2013). The consequences of choosing depend on how the options differ in value, and how readily identifiable and discriminable different options are (Egan 1975). This process becomes more challenging when the options are difficult to discriminate (Egan 1975). In such situations, animals may often need to balance the potentially high value of a focal option with their ability to definitively tell it apart from other less valuable "distractors". How animals optimize decision-making in these complex scenarios should have important consequences for a range of ecological interactions in which these situations arise, including predation and mate choice (Van Gossum *et al.* 2008; Abbott & Sherratt 2013).

Mimicry frequently forces animals to make a decision where positive and negative outcomes are difficult to discriminate from each other (Oaten, Pearce & Smyth 1975). For example, predators often face this challenge when attempting to discriminate between toxic prey and non-toxic mimics (Getty 1985). Such scenarios have been modeled in the context of predation by combining signal detection theory (Green & Swets 1988) and optimal diet theory (Stephens & Krebs 1987) in models that consider both profitability (i.e., optimization of the rate of energy or fitness gain) and

discriminability (Getty 1985; Holen 2013). Optimal diet theory determines the decisions that will optimize the rate of currency gain (e.g. fitness gain or energy gain) while signal detection theory quantifies how well an individual can discriminate between stimuli that exhibit sensory overlap (Green & Swets 1988). Holen's model, for example, explores how taste and toxicity of taste-mimics affects predators' sampling strategies and preferences (Holen 2013). Holen finds that mimicry systems are more profitable to predators when models are so toxic that predators can discriminate between models and mimics based on taste. Getty's model (1985) describes the circumstances under which predators should pursue palatable mimics of toxic species when alternative, non-mimetic (i.e., readily discriminable) options are available. Getty finds that predator's preference for mimics increases as the abundance of mimics in the community increases and as discriminability increases. Getty's model also reveals that preference for mimics should be higher when unpalatable models are less toxic and when there are fewer alternative options available in the community.

Theoretical models to date have focused on optimal decision-making in the context of predation on mimetic and non-mimetic prey. However, conceptually similar situations often arise in the context of mate choice when prospective mates are polymorphic. In a wide variety of taxa, one sex is polymorphic, and the opposite sex must adopt a strategy for optimally searching for mates (Roulin & Bize 2007). Further, in many of these polymorphic species, at least one morph resembles a non-mate stimulus (e.g., 'andromorph' females in damselflies, Gering 2017, 'alba' females that resemble heterospecifics in *Colias* butterflies, Limeri and Morehouse 2014). Mate searching individuals might have a hard time discriminating between conspecific potential mates

and non-mate stimuli because they resemble each other (mimicry) or due to limited sensitivity of the relevant sensory system. Mimicry may be caused by a morph mimicking toxic species to avoid predation (e.g. *Papilio dardanus* females Cook et al. 1994), or mimicking non-mates to avoid excessive mating attention (e.g. damselflies Gering 2017). Alternatively, even when potential mates do not resemble non-mate stimuli very closely, animals acting on limited sensory systems may still have a difficult time discriminating between them.

While many of the same insights from the models of Getty (1985) and Holen (2013) are generalizable to a mating context, considering these reproductive scenarios highlights particular issues that would benefit from extensions of these models. For example, when a mate-searching individual encounters a morph whose identity is uncertain, it may take time and/or effort for the individual to carefully evaluate the phenotype before deciding how to act (recognition cost). However, current models assume that individuals make cost-free, instantaneous decisions about how to act when encountering prospective mates or prey. In our experience studying mate-searching butterflies, this is not the case. Rather, males often investigate stimuli that resemble prospective mates, incurring a small time and energetic cost, which is presumably outweighed by the potential benefit should the stimulus indeed turn out to be a mate. The extent to which effort spent attempting to discriminate options impacts optimal decision-making strategy could benefit from explicit modeling consideration.

Sex-limited polymorphisms raise the question of how multiple morphs are maintained in a population over time. The long term maintenance of a polymorphism implies that the morphs have roughly equal fitness over evolutionary periods, yet how

this is achieved and the effects of sexual selection on polymorphism maintenance are often poorly understood (Roulin & Bize 2007). When selection imposed by mate choice is frequency-dependent, mate choice can lead to balanced polymorphisms. However, when mate choice is fixed, spatiotemporal variation or countervailing selection must be invoked to explain polymorphism persistence. Therefore, understanding optimal mate choice behavior and how mate choice affects morph fitness is a key component of understanding the maintenance of sex-limited polymorphisms. Modeling optimal mate choice and the resulting fitness consequences for each morph can provide insight to how this form of intraspecific diversity is maintained over time.

To address these questions, we extended the model presented by Getty (1985) to consider recognition time. We then built a model to estimate how mate preferences affects the fitness of morphs. We then apply the insights of these models to better understand a specific polymorphism, the widespread ‘alba’ polymorphism in the butterfly, *Colias philodice*.

5.2 MODEL DESCRIPTION

Getty’s model (1985) combines elements of signal detection theory and optimal foraging theory. In our application, this model simulates contexts where a mate-searching individual (decision-maker) must choose between multiple potential mates where there exists some perceptual overlap between one potential mate and a non-mate stimulus. We will refer to the conspecific morph that is difficult to discriminate from non-mate stimuli as the “ambiguous morph,” the non-mate stimulus as the “distractor,”

and the conspecific morph that does not resemble the distractor as the “discriminable morph.” We refer to an individual that is either an ambiguous morph or a distractor as an “uncertain phenotype.”

In the language of signal detection theory, an event where a mate searching individual correctly identifies and pursues an ambiguous morph is termed a “hit” where p(Hit) = p(pursues | ambiguous morph). Conversely, when a mate-searching individual mistakenly pursues a distractor, it is termed a “false alarm” where p(False Alarm) = p(pursues | distractor). The relationship between p(Hit) and p(False Alarm) is constrained by how well mate-searching individuals can discriminate between the ambiguous morph and the distractor. This constraint is captured in the Receiver Operator Characteristic (ROC) (Egan 1975). Following Getty (1985), we use a simple power-law relationship to define the ROC: $p(\text{Hit}) = p(\text{False Alarm})^k$ where k is a variable that describes how well mate-searchers can discriminate between ambiguous morphs and distractors (Fig. 13). A discriminability value of k = 1 indicates that mate-searchers have no ability to discriminate between ambiguous morphs and distractors (mimicry is perfect) and k=0 indicates that mate-searchers can perfectly discriminate between the ambiguous morphs and distractors. At k=1, mate-searchers are randomly pursuing uncertain phenotypes and p(Hit) = p(False Alarm). At k=0, mate-searchers have no difficulty discriminating and choosing only ambiguous morphs and p(Hit) is unrelated to p(False Alarm). At intermediate discriminability, mate-searchers are constrained to operate somewhere along the ROC curve. Varying k influences how p(Hit) varies with p(False Alarm) where lower k values allow a mate-searcher to earn a higher p(Hit) at lower p(False Alarm) rates (Fig. 13).

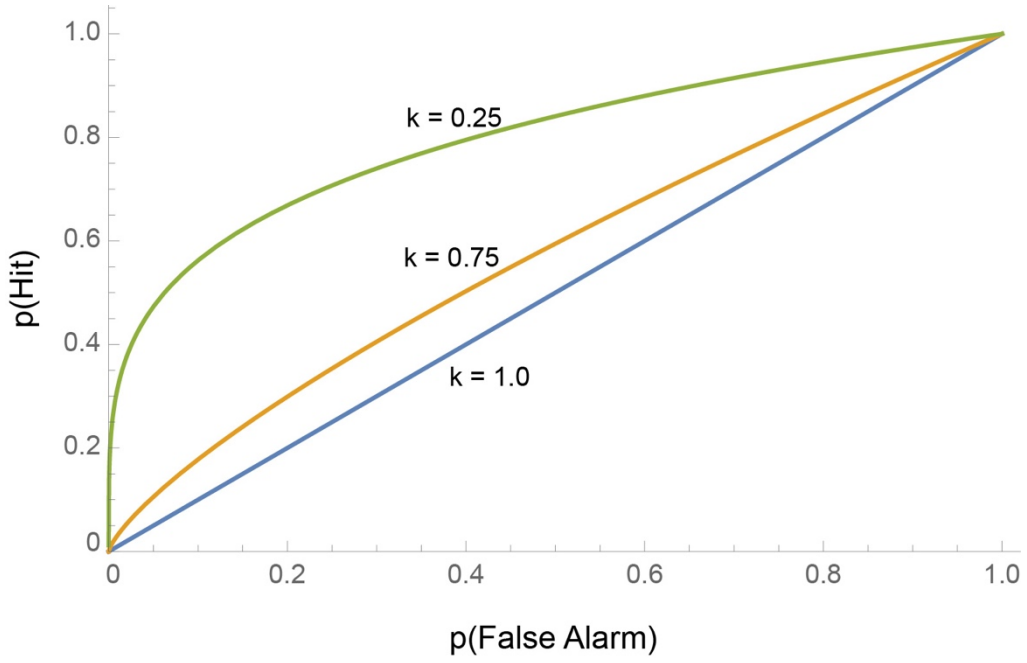


Figure 13. Receiver Operator Characteristic curves

Receiver Operator Characteristic (ROC) curves at different discriminability values (k).

Mate-searchers may adopt a risky mating strategy by pursuing uncertain phenotypes frequently, achieving high p(Hit) and simultaneously high p(False Alarm). Conversely, a more conservative strategy would be to reduce the p(False Alarm) by rarely pursuing any uncertain phenotypes, and consequently also achieving a low p(Hit). The goal of this model is to discover where along this curve the optimal behavior lies for mate-searchers under different environments, and how these decisions influence polymorphism maintenance.

For this model, let there be two morphs, ambiguous morph *a* and discriminable morph *b*, where morph *a* provides a greater mating value than morph *b*, but is also

difficult to discriminate from a distractor, type *x*. Morph *b* is always pursued with probability $P_b = 1$, but morph *a* and type *x* are pursued with probabilities P_a and P_x, respectively. P_a and P_x are related to each other through the power-law Receiver Operator Characteristic: $P_a = P_x^k$ where k is the discriminability constant. The optimal behavior will be determined by finding the p(Hit) values and corresponding p(False Alarm) values that maximize the following equation which gives the long term rate of fitness gain to a decision-maker (Getty 1985):

Equation 1. $$W/t = S\frac{[N_b*P_b*V_b+N_a*P_a*V_a+N_x*P_x*V_x]}{1+S[N_b*P_b*H_b+N_a*P_a*H_a+N_x*P_x*H_x]}$$

where the variables are described in table 3.

Table 3. Variable descriptions

Descriptions of variables in Equation 1 and the basic conditions of the model.

Variable	Description	Basic Condition
W	Fitness of mate-searcher	
t	Time	
N_a	Number of ambiguous morph individuals per unit area.	10
N_b	Number of discriminable morph individuals per unit area.	10
N_x	Number of distractors per unit area.	10
V_a	Mating value of the ambiguous morph.	1

V_b	Mating value of the discriminable morph.	0.9
V_x	Mating value of distractors.	0.5
H_a	Handling time of the ambiguous morph.	1
H_b	Handling time of discriminable morph.	1
H_x	Handling time of distractors.	1
P_a	Probability the mate-searcher pursues an ambiguous morph. P_a = p(Hit)	P_x^k
P_b	Probability the mate-searcher pursues a discriminable morph.	1
P_x	Probability the mate-searcher pursues a distractor. P_x = p(False Alarm)	
k	Discriminability value indicating how readily mate-searchers can discriminate between ambiguous morphs and distractors (*a* and *x*). K ranges between 0 and 1 where low k indicates greater discriminability and k=1 indicates that uncertain phenotypes cannot be discriminated at all (they are identical).	0.75
S	Mate-searcher search speed (area searched / time)	1

The optimal value of P_x (and consequently P_a) are found by setting $d(W/T)/d(P_x)$ equal to zero and ensuring that the location found is a maximum rather than a minimum

by checking that the second derivative is negative. Differentiating equation 1 with respect to P_x and solving yields:

$$0 = \frac{k * N_a * P_x^{-1+k} * V_a + N_x * V_x}{1 + H_b * N_b * P_b * + H_x * N_x * P_x^k}$$

$$- \frac{\left(H_x * N_x + H_a * k * N_a * P_x^{-1+k}\right) * (N_a * P_x^k * V_a + N_b * P_b * V_b + N_x * P_x * V_x)}{(1 + H_b * N_b * P_b + H_x * N_x * P_x + H_a * N_a * P_x^k)^2}$$

This basic model is identical to the one employed by Getty to understand predator diet choice between mimic and alternative prey options where there is only one alternative option (Getty 1985). First, we use the basic model to understand optimal behavior under a variety of different circumstances by varying key parameters. Next, we relax the assumption that mate-searchers should always pursue discriminable morphs to examine if and under what conditions a decision-maker should reject the discriminable morph. Then, we modify the model to recognition costs and explore the effect of recognition costs on optimal mate-choice strategy. Finally, we model the fitness of the two morphs to understand how mate choice strategy influences polymorphism maintenance.

5.3 EXPLORE PARAMETER SPACE

By varying a number of parameters, we were able to approach several important biological questions. First, we were interested in understanding the role of sensory limitations on decision making. Organisms vary in their ability to tell stimuli apart, and this may have an impact on their decision-making strategies. Further, we also explored

how the abundance of ambiguous morphs and distractors in the community affect optimal mate choice. If the frequency of ambiguous morphs and distractors in the community influences decision-making, then frequency-dependence may be acting on the system. This is important to examine because frequency dependent mate choice can result in the stable maintenance of a polymorphism if it inflicts negative frequency dependent selection on the morphs. Therefore, we examined how variation in discriminability and the ratio of ambiguous morphs to distractors in the community affects optimal mate choice (Fig. 14).

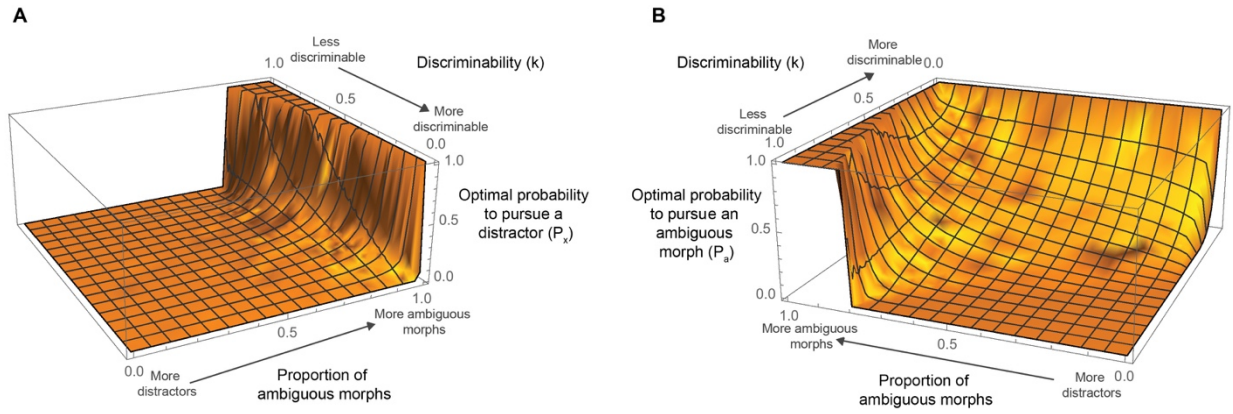


Figure 14. Optimal behavior over discriminability and proportion of ambiguous morphs.

The optimal probability to pursue a distractor (A) and the optimal probability to pursue an ambiguous morph (B) over a range of discriminability values and proportion ambiguous morphs in the community.

As discriminability increases, the probability that a mate-searcher pursues a distractor (P_x) decreases (Fig 14A) while the probability that a mate-searcher pursues

an ambiguous morph (P_a) increases (Fig. 14B). When discriminability is low (high k), mate-searchers avoid uncertain phenotypes (both P_a and P_x tend to be lower), except for when the proportion of ambiguous morphs is high. Conversely, when discriminability is high (low k), mate-searchers are able to target ambiguous morphs and avoid distractors (high P_a and low P_x, Fig. 14). When distractors make up most of the community, mate-searchers are unlikely to pursue any uncertain phenotypes (Fig. 14). This means that as the relative abundance of ambiguous morphs increases, a mate-searcher should be riskier and pursue uncertain phenotypes more often because the likelihood that an uncertain phenotype is an ambiguous morph is higher. Further, the effects of the relative abundance of ambiguous morphs in the community and the discriminability between ambiguous morphs and distractors on the probability to pursue an ambiguous morph (P_a) are qualitatively additive (Fig. 14). When discriminability is high, mate-searchers pursue ambiguous morphs over a wider range of community compositions (Fig. 14B). That is, if mate-searchers are able to discriminate ambiguous morphs from distractors, they will pursue ambiguous morphs even when they are less common in the community because they can be reliably identified. Conversely, when discriminability is low, mate-searchers should adopt a switch-point strategy based on the abundance of ambiguous morphs in the community such that when ambiguous morphs are very common, a mate-searcher should always pursue uncertain phenotypes. This is because when ambiguous morphs are abundant in the community and distractors are rare, the a priori probability that an uncertain phenotype will turn out to be an ambiguous morph as opposed to a distractor is high. This switch-point strategy occurs because when discriminability is low, mate-searchers cannot reliably determine

whether an uncertain phenotype is an ambiguous morph or a distractor, and must decide whether to pursue the uncertain phenotype or not based purely on probability of encountering an ambiguous morph based on their relative abundance in the community.

5.4 OPTIMAL PROBABILITY TO PURSUE A DISCRIMINABLE MORPH

The base model uses a prediction of the classical optimal diet model called the zero-one rule, where prey should be always attacked, or never attacked upon encounter. Thus far, the model has operated under the assumption that mate-searchers should always pursue discriminable morphs when encountered ($P_b = 1$). However, in the context modeled here, where discriminable morphs are less profitable than ambiguous morphs, there may be situations where it is optimal for mate-searchers to avoid the ambiguous morphs or adopt partial preferences, such as when ambiguous morphs are very common and readily discriminable. Therefore, we used Equation 1 to optimize for both P_a and P_b under the basic conditions and a range of discriminability (k) and abundance of ambiguous morphs (Fig. 15). Modeling reveals that mate-searchers should not pursue discriminable morphs when discriminability is extremely high (mate-searchers have very little difficulty discriminating between “ambiguous” morphs and distractors) and that this effect is mediated by community composition. As the abundance of ambiguous morphs in the community increases, decision-makers will reject discriminable morphs at lower discriminability values. We find this pattern because here ambiguous morphs provide a higher mating value than discriminable

morphs, so when mate-searchers can readily identify abundant “ambiguous” morphs, it is optimal to only pursue the higher value “ambiguous” morphs.

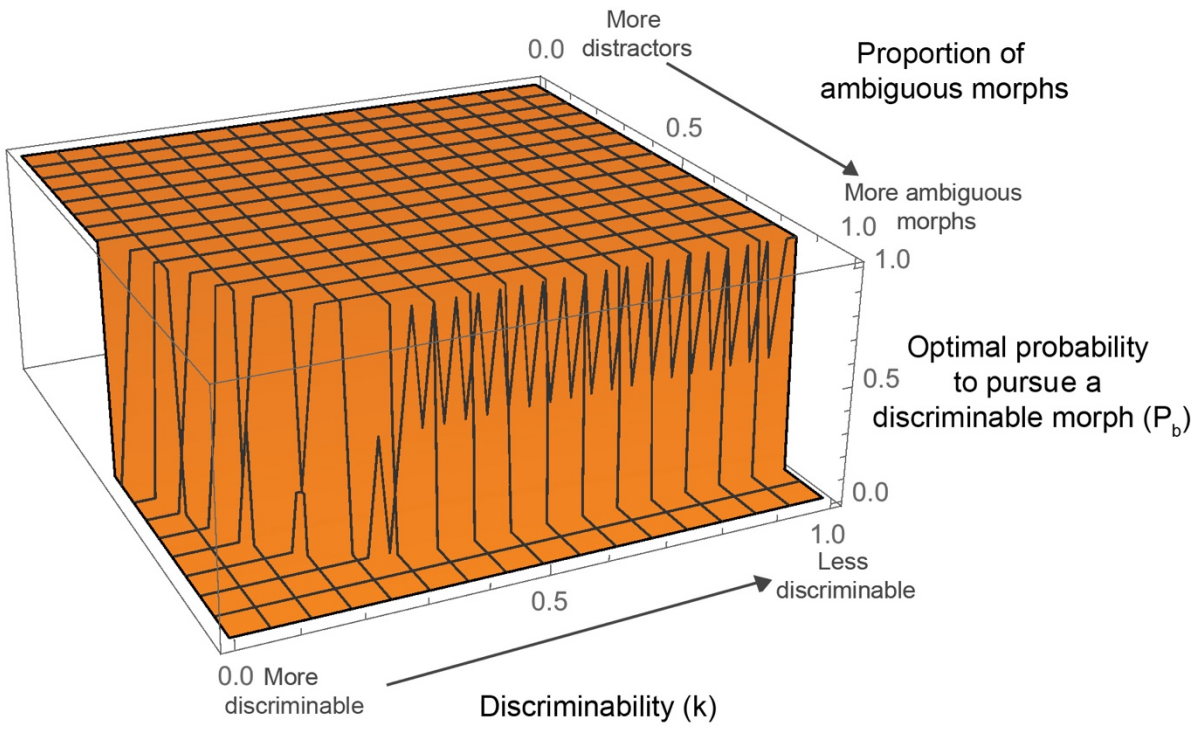


Figure 15. Optimal probability to pursue discriminable morphs

Optimal probability to pursue discriminable morphs over discriminability and proportion of ambiguous morphs

5.5 RECOGNITION TIME

One simplifying assumption in this model is that spotting and identifying the different outcomes occurs instantaneously. However, it is likely that in many situations, identifying potential outcomes requires time to evaluate the identity of an outcome, and

perhaps even requires approach for more careful inspection, particularly when some outcomes are difficult to discriminate between. These investigation behaviors are likely to carry at least a time cost, and perhaps an energetic cost as well when investigation requires approaching or chasing morphs or distractors. These costs may have significant implications for the optimal strategy. Therefore, we modified the model to explicitly incorporate a recognition time in the following way. Let R represent recognition cost, a sunk cost mate-searchers pay by investigating and evaluating the identity of a morph or distractor. Incorporating R into Equation 1 above yields the long term rate of fitness gain:

Equation 2: $$W/t = S\frac{[N_b*P_b*V_b+N_a*P_a*V_a+N_x*P_x*V_x]}{1+S[N_b(P_b*H_b+R)+N_a(P_a*H_a+R)+N_x(P_x*H_x+R)]}$$

When R=0, the model is identical to the base model. Equation 2 assumes that discriminable morphs, ambiguous morphs, and distractors all require the same recognition cost to identify. We also ran models where the recognition cost of discriminable morphs is lower than the recognition costs of uncertain phenotypes because they are more discriminable and likely take less effort to identify, and this change did not affect the overall patterns reported here. When recognition costs are low or non-existent, mate-searchers should only pursue an ambiguous morph when they are abundant in the community (Fig. 16). As recognition costs increase, mate-searchers should pursue ambiguous morphs at lower densities (Fig. 16). Therefore, higher recognition costs cause the optimal mate-searcher to pursue ambiguous morphs in a

wider range of communities. Consequently, high recognition costs cause the optimal strategy to become riskier. This indicates that when decision-makers experience a high cost for evaluating every potential option encountered, they should become less selective because the proportional cost associated with pursuing any given type decreases since a cost is paid no matter which type is pursued.

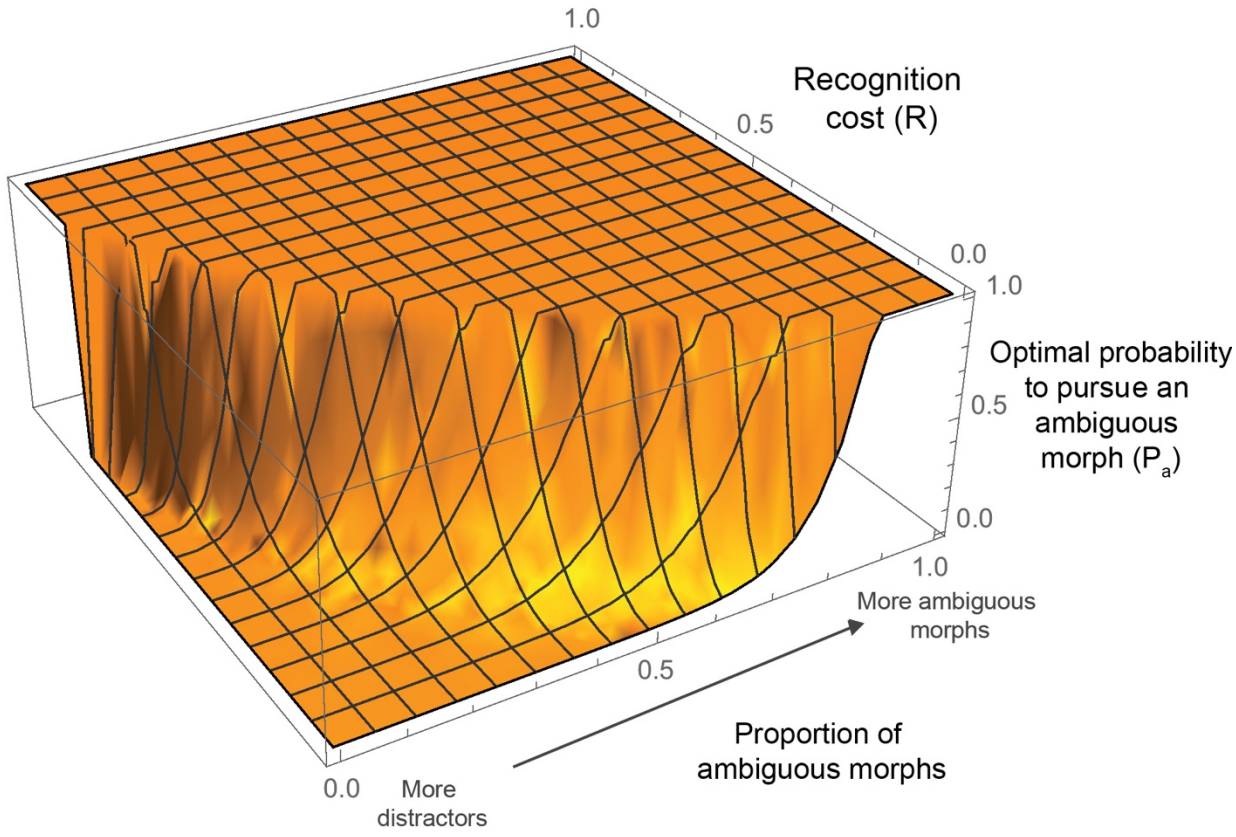


Figure 16. Optimal behavior over a range of recognition costs

Optimal probability to pursue an ambiguous morph (P_a) over a range of recognition costs and proportion of ambiguous morphs in the community.

5.6 MODELING MORPH FITNESS

We also investigated the effect of a mate-searcher's decisions on the relative fitness of the morphs in order to understand when a polymorphism would persist. In order to accomplish this, we created a set of equations to model morph fitness. Assuming that the fitness of each morph is only a function of fecundity and amount of attention from mate-seekers, fitness can be modeled using the following equations:

Equation 3. $W_a = (1 - |(O_a - P_a)|) * F_a$

Equation 4. $W_b = (1 - |(O_b - P_b)|) * F_b$

where W_a is the fitness of ambiguous morph, O_a is the optimal amount of mating attention for ambiguous morphs, P_a is the actual amount of mating attention that ambiguous morphs experience, F_a is the relative fecundity of ambiguous morphs, W_B is the fitness of discriminable morphs, O_b is the optimal amount of mating attention for discriminable morphs, P_b is the actual amount of mating attention that discriminable morphs experience, and F_b is the relative fecundity of discriminable morphs.

The optimal amount of mating attention will vary depending on the situation being modeled. Specifically, the value of O_a and O_b will depend to some extent on population density. For example, in low-density populations, the optimal amount of mating attention is likely to be high because mates may be scarce such that every opportunity to mate

improves fitness. Conversely, in a high-density population, the optimal amount of mating attention may be intermediate because too few interactions result in reduced mating rates yet too many interactions may impose harassment costs, such as preventing females from feeding or ovipositing (Gilchrist & Rutowski 1986). Here we model the consequences of both high and low values of O because they mimic these two ecological scenarios (e.g. high vs low density populations). We also model the assumption that the ambiguous morph has a higher fecundity than the discriminable morph in order to represent the tradeoff between value and discriminability. If ambiguous and discriminable morphs had equal fecundity, mate-searchers would always pursue the discriminable morph and there would be no difficult decision requiring modeling insight. Therefore, we set the fecundity values to $F_a = 1$ and $F_b = 0.9$. The values for P_a and P_b are determined using Equation 2 above, optimizing for both P_a and P_b. Using these conditions, we explored how the fitness of the ambiguous and discriminable morphs vary as the proportion of ambiguous morphs and distractors varies (Fig. 17).

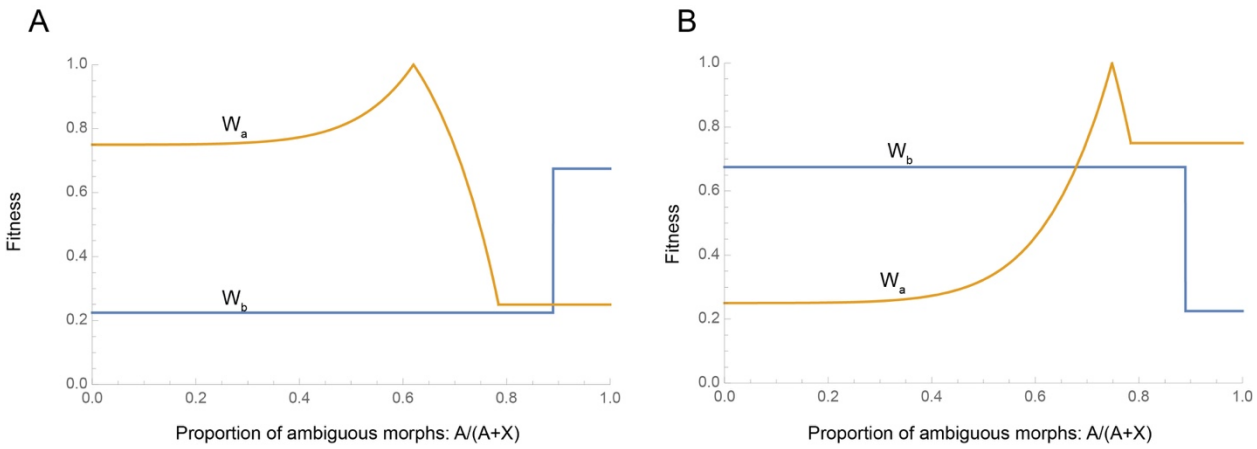


Figure 17. Fitness of ambiguous and discriminable morphs

Fitness of ambiguous morphs (W_a) and discriminable morphs (W_b) over a range of proportion of ambiguous morphs when optimal amount of attention from decision-makers is low (O_a=O_b=0.25) (A) and high (O_a=O_b=0.75) (B).

When the optimal amount of attention is low, the ambiguous morph is more fit in most communities because the ambiguous morph receives very little mating attention in most communities. The ambiguous morph only becomes less fit than the discriminable morph when the proportion of ambiguous morphs is very high, because mate-searchers only pursue ambiguous morphs when they are very common (Fig. 17A). When the optimal amount of mating attention is high, the discriminable morph is more fit under most communities because the discriminable morph receives more mating attention in most communities. The ambiguous morph becomes more fit when ambiguous morphs become common in the population relative to distractors because when ambiguous morphs are common they receive more mating attention (Fig. 17B). This indicates that the population density of distractors can have a significant impact on the relative fitness of the two morphs by shifting mate preferences. Since male mate preferences impact

female morph fitness, the population density of distractors would also influence the stability of the polymorphism. Further, the optimal amount of mating attention is affected by the density of the mate-searchers, indicating that female morph fitness and polymorphism maintenance would also be affected by the population density of the polymorphic species as well. Therefore, the community composition plays an important role in determining whether the polymorphism is stable over evolutionary periods. This suggests that factors that influence the relative density of the mate-searchers and distractors can cause the polymorphism to persist or be lost by influencing the relative fitness of the morphs.

5.7 OPTIMAL MATE CHOICE IN *COLIAS* BUTTERFLIES

This model could be used to understand any situation in which a decision-maker is choosing between options that vary in discriminability and value. For example, we will apply this model to understand how male *Colias* butterflies select mates and how male mate preferences influence the fitness of multiple female color morphs.

The Alfalfa Butterfly, *Colias philodice*, is characterized by a female-limited color polymorphism where females are either white (termed the ‘alba’ morph) or yellow (termed the non-‘alba’ morph). This polymorphism is widespread throughout the Coliadinae sub-family of butterflies, yet the factors maintaining it are not fully understood (Limeri & Morehouse 2016). The current best-supported hypothesis is that the polymorphism is being maintained through countervailing selection arising from a

resource advantage available to ‘alba’ females that is offset by an attractiveness cost during mate choice (Nielsen & Watt 2000).

‘Alba’ females enjoy a developmental advantage over non-‘alba’ females as a direct consequence of their color phenotype. The ‘alba’ phenotype requires reduced pterin pigment investment, resulting in lower expenditures of nitrogen-rich precursors to this pigment pathway during development (Watt 1973). As a result, during pupal development, ‘alba’ females are able to redirect some nitrogen (0.4mg of GTP) away from pigment production to other developmental processes (Watt 1973). This redirection of an essential developmental resource, nitrogen, allows ‘alba’ females to develop more quickly in the pupal stage and emerge from pupae with more mature eggs and fat reserves than non-‘alba’ females do (Graham *et al.* 1980). Therefore, ‘alba’ females are of potentially higher reproductive value to males because they are more fecund, and are more likely to lay eggs more quickly after their first mating post-eclosion. However, this is counteracted by their visual similarity to other white butterflies present in the community, namely ‘alba’ females of a congener *C. eurytheme* and white pierine butterflies (e.g., *Pieris rapae* (Limeri & Morehouse 2014). Modeling of the visual system of male *Colias* butterflies indicates that males may have a difficult time visually discriminating between conspecific ‘alba’ female and heterospecific ‘alba’ females (Limeri & Morehouse 2014) and field observations report that *Colias* males spend up to 11% of their interactions with butterflies courting pierines (Nielsen & Watt 2000). Hybrid matings between *C. philodice* and *C. eurytheme* are common and produce offspring that have reduced fitness (Grula & Taylor 1980). Male mating preferences are important because they drive how frequently hybrid matings occur due to the fact that hybrid

matings almost exclusively occur when females are freshly eclosed and are unable to reject male attention while their wings expand and dry (Taylor 1972; Silberglied & Taylor 1978). Males invest a large amount of resources into each mating by producing a large, protein and nutrient-rich spermatophore that is nutritionally costly for males to donate (Boggs & Watt 1981). Therefore, mistaking a heterospecific female for a conspecific female would cause a male to invest limited resources into a mating that produces fewer fertile offspring, thereby imposing a fitness cost to the male. *Colias* males are not able to mate with pierine females so do not risk investing resources in a hybrid mating. However, there are almost certainly time and energy costs associated with mistakenly identifying pierid butterflies as conspecific 'alba' females and courting them.

Previous work on this system has suggested that males prefer to mate with the easily discriminable non-'alba' female and that male mate preferences may play a role in maintaining the 'alba' polymorphism (Nielsen & Watt 2000). Male mate preference can have significant positive or negative effects on female fitness (Nielsen & Watt 1998). Females use the nutrients derived from spermatophores for crucial functions such as producing eggs and somatic cell maintenance (Boggs & Watt 1981). Consequently, *Colias* females mate multiple times during their life span and rely on nutrients from the spermatophore because their natural diet is low in protein (Boggs & Watt 1981). Therefore, being disfavored by males could have a significant negative impact on fitness if it results in a female having delayed or reduced access to these important nutritional resources (Nielsen & Watt 1998). Conversely, too much male attention may also adversely affect female fitness. Constant male harassment results in less time for nectaring and ovipositing (Nielsen & Watt 1998). Furthermore, rejecting

male courtship attempts involved an ‘ascending flight’ that can exceed 40m altitude, imposing a significant energy and time cost to females rejecting males (Rutowski 1978). Dispersal from the natal field to avoid male harassment has been observed in high density populations and is costly to females because dispersing prevents females from nectaring or ovipositing while in flight (Gilchrist & Rutowski 1986). The overall impact of male attention on female fitness likely depends on the population density. In low-density populations, a lack of attention would negatively impact female fitness because mates are scarce and increased attention would not have a strong effect. Conversely, in high density populations, being disfavored would result in lower harassment but disfavored females would still be able to readily find a mate when necessary.

If reproductive interference between conspecific ‘alba’ females and heterospecific white butterflies affect male mate preferences, then the relative abundance of these types in the community should be an important factor influencing selective pressures on morphs via mate preferences (Ley & Watt 1989; Watt *et al.* 1989). This community-dependent mate preference would then influence the relative morph stability within a population over time. Previous modeling efforts on related *Colias* species consider how the number of courtship interactions influences the fitness of ‘alba’ and non-‘alba’ female morphs (Nielsen & Watt 2000). Neilsen and Watt’s model (2000) revealed that ‘alba’ females can lose up to 38% of their total lifetime fecundity due to reproductive interference depending on the absolute density of heterospecific white butterflies and males. The model we propose here builds on this work by considering optimal male mate preferences and investigating the effects of variation in relative rather than absolute density of heterospecific white butterflies. Furthermore, we consider

heterospecific *Colias* ‘alba’ females which can result in hybrid matings as the heterospecifics rather than pierines, which cannot produce hybrid matings.

In the context of the model presented here, the decision-makers are *Colias philodice* males who are choosing to court either: non-‘alba’ females (the discriminable morph), conspecific ‘alba’ females (the ambiguous morph), or heterospecific ‘alba’ females (the distractors). We will refer to the latter two uncertain phenotypes together as “white butterflies.” For simplicity, we will consider only heterospecific ‘alba’ females and not pierines as the distractors. The base values of the parameters for the *Colias* system are outlined in Table 4 below:

Table 4. Variable descriptions and basic conditions of the model applied to *Colias*

Variable	Description	Basic Condition
N_a	Number of conspecific ‘alba’ females per unit area.	10
N_b	Number of conspecific non-‘alba’ females per unit area.	10
N_x	Number of heterospecific ‘alba’ females per unit area.	10
V_a	Mating value of conspecific ‘alba’ females.	1
V_b	Mating value of conspecific non-‘alba’ females.	0.85
V_x	Mating value of heterospecific ‘alba’ females.	0.5
H_a	Handling time of conspecific ‘alba’ females.	1

H_b	Handling time of conspecific non-‘alba’ females.	1
H_x	Handling time of heterospecific ‘alba’ females.	1
P_a	Probability the male courts a conspecific ‘alba’ female. P_a = p(Hit)	P_x^k
P_b	Probability the male courts a conspecific non-‘alba’ female.	1
P_x	Probability the male courts a heterospecific ‘alba’ female. P_x = p(False Alarm)	
k	Discriminability value indicating how readily males can discriminate between conspecific and heterospecific ‘alba’ females (A and X).	0.5
R	Recognition cost incurred by spending time and/or energy to investigate and identify a potential outcome	0.01
S	Male search speed (area searched / time)	1

The mating value of heterospecific ‘alba’ females is set to half the mating value of a conspecific ‘alba’ female because the female offspring of a *C. philodice* male x *C. eurytheme* female mating are almost always sterile whereas the males are generally fertile (Grula & Taylor 1980). The mating value of a conspecific non-‘alba’ female is lower than the conspecific ‘alba’ female because the ‘alba’ females have on average 25% more body fat than non-‘alba’ females, develop 2-4% more quickly than non-‘alba’ females (depending on the climate), and emerge with on average 28% more eggs than

non-‘alba’ females (though only in cold climates) (Graham *et al.* 1980). This parameter would need to be modified depending on the climate in question because the fitness difference between ‘alba’ and non-‘alba’ females is larger in colder climates. Here we use a relative fitness value for non-‘alba’ females of 0.85 to approximate an intermediate climate. We set recognition cost equal to 0.01 because male *Colias* are observed to frequently fly out of their way to approach and investigate females, and even other males, in the field. This behavior indicates that it may be necessary for *Colias* males to approach and investigate every butterfly they encounter to determine its identity and decide whether or not to pursue it further. Approaching butterflies in this way uses up flight time and energy. Approaching and investigating butterflies takes only a few seconds which is very short relative to the total mating (handling) time, which typically lasts about 45 min (Gilchrist & Rutowski 1986). Therefore, we use 0.01 to reflect the short duration of recognition approach relative to the overall handling time. Finally, since modeling of the male visual system reveals that conspecific ‘alba’ and heterospecific ‘alba’ females are moderately discriminable (Limeri & Morehouse 2014), we model situations over a range of moderate discriminability values ($0.25 < k < 0.75$).

Given these basic conditions, we examined how *Colias* male mate preferences should be affected by varying community compositions (Fig. 18). In most situations, males should adopt a conservative strategy and never pursue heterospecific ‘alba’ females (Fig 18A). Heterospecific ‘alba’ females should only be pursued when they are very rare in the community and conspecific ‘alba’ females are very abundant. Males should adopt a preference for conspecific ‘alba’ females that increases as the proportion of white butterflies that are conspecific ‘alba’ females in the community increases (Fig.

18B). When conspecific ‘alba’ females make up a small proportion of the white butterflies in the community, males should avoid courting all white butterflies because the probability that a white butterfly is a conspecific ‘alba’ female is low. If discriminability is high, males will be able to pursue more conspecific ‘alba’ females while avoiding heterospecific ‘alba’ females. Under most circumstances, males should always pursue conspecific non-‘alba’ females (Fig. 18C). Males should only reject conspecific non-‘alba’ females when conspecific ‘alba’ females are very common and heterospecific ‘alba’ females are very rare in the community.

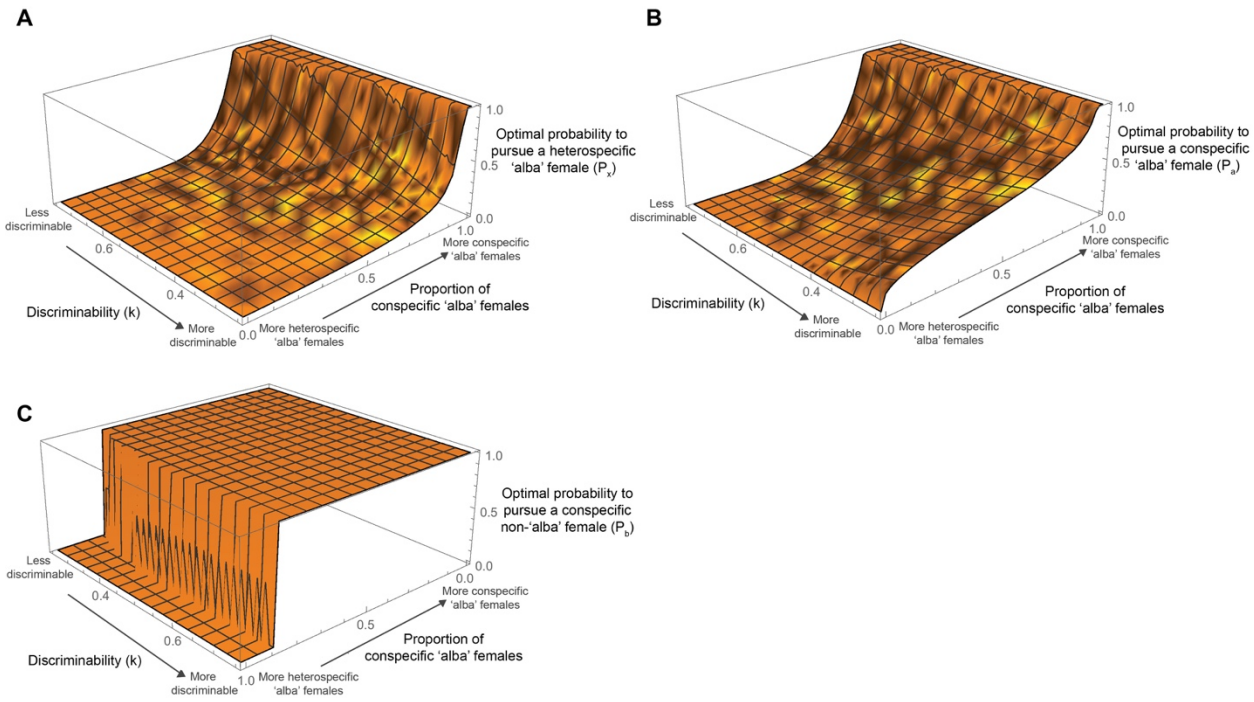


Figure 18. Male *Colias* optimal mating behavior

Optimal probability to pursue heterospecific ‘alba’ females (A), conspecific ‘alba’ females (B), and conspecific non-‘alba’ females (C) over a range of community compositions and discriminability.

5.8 *COLIAS* ‘ALBA’ AND NON-‘ALBA’ MORPH FITNESS

We then explore how these optimal male mate preferences should influence the fitness of conspecific ‘alba’ and non-‘alba’ females. Because male attention may have positive or negative effects depending on the density of the community, the optimal levels of male attention will likely vary by community. That is, in high-density populations, a lower level of male attention would be optimal whereas in low density populations, a higher level of male attention would be optimal. Therefore, we determined female fitness under both low and high density population conditions (Fig. 19). Under high density conditions, where the optimal amount of male attention is low, ‘alba’ females are more fit than non-‘alba’ females in most communities. When ‘alba’ females make up a high proportion of the white butterflies in the community, males court ‘alba’ females more frequently and non-‘alba’ females become more fit (Fig. 19A). Under low density conditions, where the optimal amount of male attention is high, ‘alba’ females are more fit when they make up over 60% of the white butterflies in the community, and non-‘alba’ females are more fit when the ‘alba’ females are less abundant (Fig. 19B). Ultimately, this indicates that morph fitness, and therefore polymorphism maintenance, is dependent on not only density of males in the population, but also on the population density of other coflying *Colias* species.

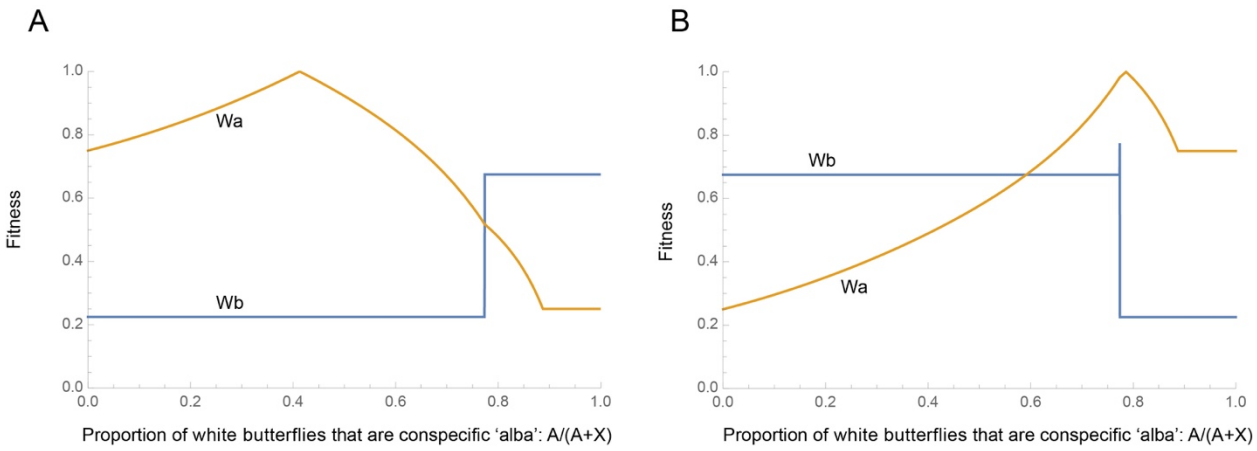


Figure 19. 'Alba' and non-'alba' morph fitness

Fitness of conspecific 'alba' (W_a) and non-'alba' (W_b) female morphs over a range of proportion of white butterflies that are conspecific 'alba' females when optimal amount of attention from males is low (O_a=O_b=0.25) (A) and high (O_a=O_b=0.75) (B).

5.9 DISCUSSION

Optimal decision-making models have been used to describe predatory behavior in a variety of circumstances, including decisions between prey types that trade off discriminability and value (Getty 1985; Holen 2013). These types of models also have the potential to offer insight into optimal mate choice, but have been underutilized in this way. Here, we built on an existing model that combines Signal Detection Theory and Optimal Diet Theory and applied it to understand optimal mate choice and morph fitness in a polymorphic system. We then used this model to answer more specific questions about a widespread female-limited color polymorphism in *Colias* butterflies.

Exploring parameter space in the base model revealed that discriminability and the proportion of ambiguous morphs and distractors in the population significantly affect optimal mate choice strategy. When discriminability is low, mate-searchers have very little information about uncertain stimuli and are forced to adopt a switch-point strategy which depends only on the relative proportion of ambiguous morphs and distractors in the community. Mate-searchers should adopt a generally conservative strategy and avoid pursuing ambiguous morphs and distractors under most circumstances. Ambiguous morphs should only be pursued when discriminability is very high and/or ambiguous morphs are much more abundant than distractors in the community.

We then modified the base model to incorporate recognition costs which mate-searchers incur by investigating potential mates to identify them. Incorporating recognition costs shifts the optimal strategy to a riskier, less selective strategy where mate-searchers should be more willing to pursue any potential mate encountered. The higher the recognition cost, the less selective mate-searchers should become. This is because cost savings available to conservative strategies are reduced.

Next, we extended the results of optimal choice modeling to examine how mate choice influences the fitness of the morphs. We modeled morph fitness in both high-density and low-density populations because the optimal amount of mating attention likely differs between these two contexts. In low-density communities where being the favored morph is beneficial, discriminable morphs have the fitness advantage over ambiguous morphs in most situations because mate-searchers generally prefer to court the discriminable morph. Discriminable morphs are only less fit than ambiguous morphs when ambiguous morphs are very common in the population and/or discriminability is

high. The converse is true in high-density communities where being the disfavored morph is advantageous. Therefore, the population density should have a significant effect on morph relative fitness. This suggests that population density affects overall polymorphism stability.

Lastly, we apply this model to understand the ‘alba’ polymorphism in *Colias* butterflies. We find that in most contexts, males should pursue conspecific non-‘alba’ females every time they are encountered. However, when conspecific ‘alba’ females are very common and heterospecific ‘alba’ females are very rare, males should completely avoid conspecific non-‘alba’ females and only pursue conspecific ‘alba’ females. In most communities, males should adopt a relatively conservative (low risk) strategy to avoid pursuing heterospecific ‘alba’ females. However, if the proportion of white butterflies that are conspecific ‘alba’ females is very high, males should pursue all white butterflies indiscriminately because white butterflies will usually be conspecific ‘alba’ females. Together, these findings imply that the density and relative abundance of different types of butterflies in the community should drastically change male mate preferences.

5.10 CONCLUSION

The model presented here determines optimal behavior in a situation where animals have to balance value, or rate of energy gain, with discriminability. These kinds of decisions are common in predatory contexts where some palatable prey mimic toxic species. Here we have applied this model to a new ecological context: mate choice in a

polymorphic species where at least one morph resembles non-mate stimuli. Our model highlights the critical importance of the community within which predators or mate-searching individuals must make decisions. This community-based contingency is an interesting and exciting area for new research on decision-making. Further studies might benefit from considering how and under what circumstances organisms benefit from learning about community composition.

5.11 ACKNOWLEDGEMENTS

I thank Bard Ermentrout for advice and helpful discussions about this work. I am especially grateful to Nick Stanley for assistance with coding in Mathematica.

6.0 CONCLUSION

The research described above represents a number of significant advances in our understanding of the mechanisms that may underlay the evolutionary maintenance of the ‘alba’ polymorphism. I first examined the evolutionary origin and history of the ‘alba’ polymorphism using comparative approach in order to gain clues to the selective pressures acting on the ‘alba’ polymorphism. My comparative work revealed that the ‘alba’ polymorphism is likeliest to be ancestral to the Coliadinae, rather than a polymorphism that has evolved repeatedly in separate lineages. This finding suggests that questions about the ‘alba’ polymorphism would be more productively focused on reasons why it is maintained or lost rather than questions about origins. Our comparative analyses further revealed that the polymorphism has persisted in about half of the lineages (87 extant polymorphic species) in the Coliadinae, while about half have reverted to monomorphism (103 extant monomorphic species). This indicates that the selective pressures maintaining the polymorphism are stable in some lineages over a large number of generations. However, the mechanisms maintaining the ‘alba’ polymorphism in some instances are relatively unstable in the sense that there have been repeated independent losses of one of the morphs. In addition, we observe a weak bias towards non-‘alba’ fixation when a lineage reverts to monomorphism. Of the 103 monomorphic species in the Coliadinae, 40 (39%) have only ‘alba’ females and 63 (61%) have only non-‘alba’ females, indicating that the selective pressures favoring the non-‘alba’ female morph may be stronger than selective pressures favoring the ‘alba’ female morph. An alternative explanation is that one reversion in one or some lineages

to non-‘alba’ monomorphism coincidentally resulted in more daughter species than lineages that reverted to ‘alba’ monomorphism. Conversely, it is possible that species are more likely to experience conditions that favor that non-‘alba’ morph, leading to fixation of the non-‘alba’ morph. These results indicate that questions about the ‘alba’ polymorphism should focus on selective pressures acting to maintain each morphs and under which conditions ‘alba’ or non-‘alba’ female morphs are favored.

I then examined whether visual limitations may influence the selective pressures acting on the polymorphism. Modeling of color discrimination by the *Colias* visual system supported the hypothesis that sensory limitations may influence male mate preferences via reproductive interference. I showed that males are likely to have a difficult time discriminating between conspecific ‘alba’ females and heterospecific ‘alba’ females and pierid butterflies. Males may incur significant fitness costs by pursuing and courting white butterflies that turn out to be heterospecific ‘alba’ females or pierids. Consequently, it may be advantageous for males to avoid white butterflies altogether and focus mating attention on non-‘alba’ females. Thus, this visual limitation provides a functional reason for why a mating bias for non-‘alba’ females might arise. Limitations of the sensory system have been demonstrated to be an important driver of polymorphism maintenance in a range of taxa, such as damselflies and *Papilio* butterflies where some female morphs mimic males to avoid excessive male harassment (Cook *et al.* 1994; van Gossum *et al.* 2001; Sherratt 2001; Nijhout 2003). Most of these studies have relied on human vision to determine whether mates should have difficulty discriminating between potential mates and non-mates. However, this is problematic because human color discrimination can differ dramatically from the system being studied (Kelber 2006; Land

& Nilsson 2012). This study was one of the few to empirically test discriminability using a model of the visual system of the focal species, rather than human approximation (but see Schultz & Fincke 2013).

I then asked the question of whether males have biases and whether these biases are flexible and responsive to male experience. Behavioral experiments verified that males prefer to court non-‘alba’ females. The existence of a mating bias towards non-‘alba’ females corroborates previous reports that non-‘alba’ females gain more male attention and supports the long standing hypothesis that the ‘alba’ polymorphism is maintained by countervailing selection between a mating advantage for the non-‘alba’ morph and a developmental advantage for the ‘alba’ morph. Further, this study was the first to empirically evaluate whether *Colias* male preferences are fixed or plastic. Both field observations and direct experimental testing indicates that male *Colias* preferences do not vary with population morph frequency. Examples in the literature of fixed preferences in a polymorphic system are rare (Lank *et al.* 1995; Craig & Foote 2001), yet they might be widespread throughout varied animal taxa, especially when circumstances are not conducive to learning (Dukas 2006, 2008; Dukas, Clark & Abbott 2006). This finding prompts the question of whether fixed preferences in polymorphic systems are more common in nature than has been previous appreciated. Further, the lack of learning demonstrated in the Coliadinae places the Coliadinae in contrast to many polymorphic systems where learning is prominent (Hughes *et al.* 1999; van Gossum *et al.* 2001; Dukas 2006). This contrast raises questions about the value of learning and what role learning plays in maintaining polymorphisms.

Next, I built a theoretical model to determine optimal decision-making among options that differ in value and discriminability. Modeling revealed that males' ability to discriminate between conspecific 'alba' females and heterospecific white butterflies should significantly affect their mate preferences. When discriminability is poor, males should avoid all white butterflies because they are uncertain about their identity. This result supports the argument that the visual limitation described in section 2 could result in a mate preference for non-'alba' females. Further, I modified the model to include costs associated with recognizing and identifying potential mates. When identifying potential mates is costly, the optimal mating strategy becomes less selective. Thus, the more time and energy it takes to identify potential mates, the more indiscriminately males should court potential mates.

Finally, I also modeled the effect of male preferences on female morph fitness and found that male preferences can play a role in overall polymorphism maintenance by impacting female morph fitness. The overall density and relative proportion of conspecific 'alba' and heterospecific 'alba' females should significantly affect male preferences. That is, the population density and community composition should have a drastic effect on mate preference. However, field observations and experimental data indicate that males do not modify their preferences based on the community composition. There are several potential explanations for this discrepancy. One explanation is that the community composition is so variable that it is not possible for male preferences to track community composition. This could be due to too much migration between field populations resulting in communities that are too variable for learning to be helpful (Dukas 2008; Dunlap & Stephens 2009). A further possibility is

that community composition of local sub-populations are steady over larger periods of time than was captured by these field observations and that male preferences evolve to match average community composition over larger evolutionary periods of time. Finally, it's possible that the model does not include one or more key parameters that are important in this system. Further work could extend the model by including parameters such as likelihood a female is receptive to mating or refractory period between matings.

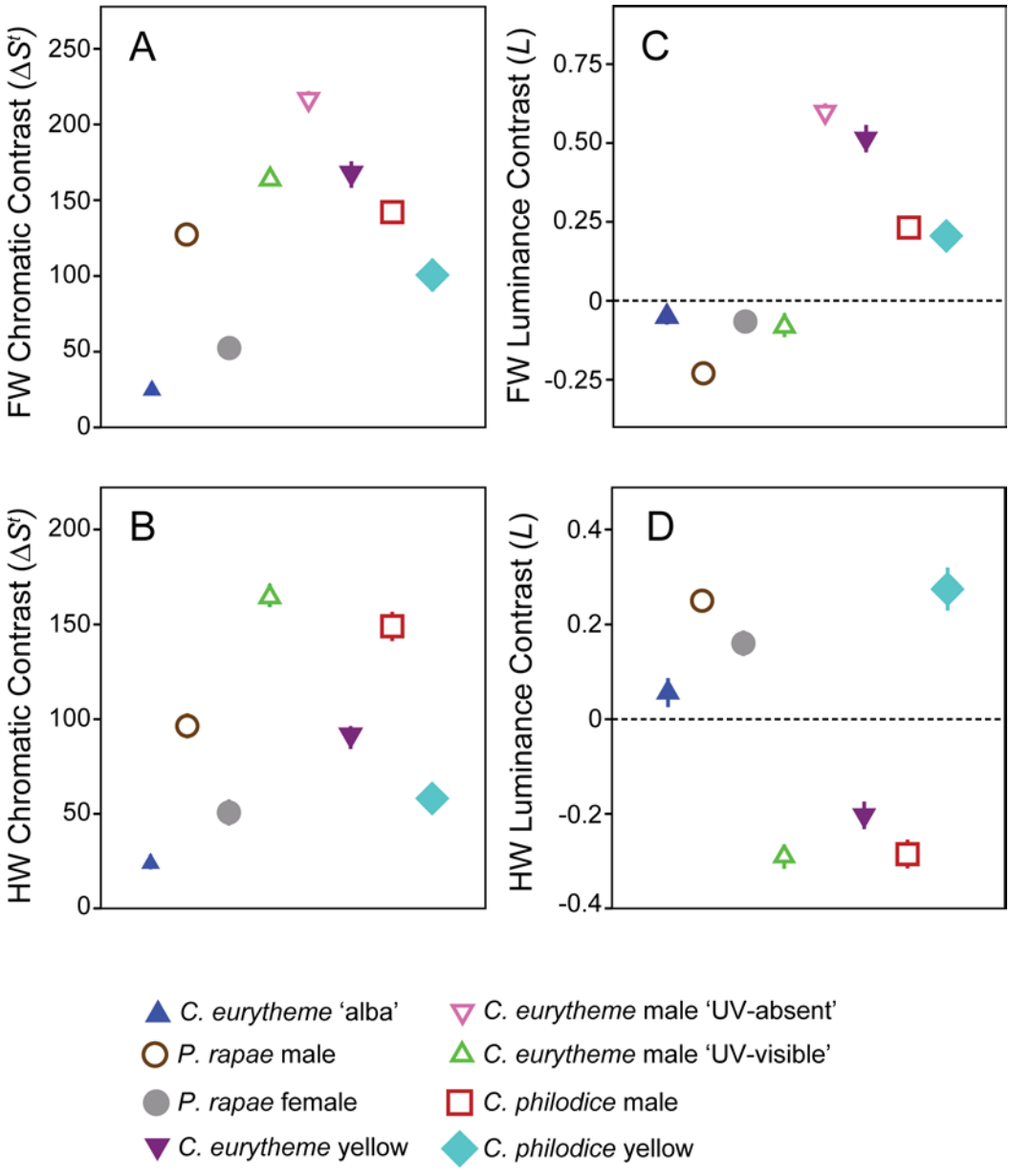
Together, my work sheds light on how the 'alba' polymorphism is maintained over time. Modeling of the male visual system and behavioral data indicate that sensory biases may be one reason that males possess a fixed mate preference for non-'alba' females. Theoretical modeling suggests that this preference could have a significant impact of the fitness of the 'alba' and non-'alba' female morphs. Further, my comparative work reveals that the polymorphism has been lost in approximately half of the Coliadinae species, which suggests the selective regime maintaining the polymorphism has been frequently disrupted. Together, these results suggest that the 'alba' polymorphism may be maintained by countervailing selection where male mate preferences, influenced by visual limitations, must be countered by some other selective pressure. The nature of this countervailing selective force is not wholly understood. One possibility is that 'alba' females have a fecundity advantage over non-'alba' females. However, it is unclear whether this advantage exists across the entire Coliadinae sub-family and whether it is capable of countervailing the selective pressures imparted by male mate preferences. In fact, fecundity differentials are likely to vary in different environments. Previous research has found that the developmental differences between 'alba' and non-'alba' females are exaggerated in cold climates and diminished in warm

climates (Graham *et al.* 1980). My dissertation did not address the fecundity differential and this remains to be further explored. Further, countervailing selection generally requires fluctuating selection where the selective pressures vary temporally and/or spatially. Further research could explore how the selective pressures acting on the 'alba' polymorphism vary.

There are few well-supported examples of countervailing selection maintaining a polymorphism in the literature. Rather, many polymorphisms are maintained by negative frequency dependent selection, such as guppies (Magurran & Ramnarine 2004) where females prefer rare males, and damselflies, where the rare female morph suffers less harassment from males (Van Gossum *et al.* 1999). Further, polymorphic systems often favor learned mate preferences, (Van Gossum *et al.* 1999; Dukas 2008) yet my data demonstrate that male *Colias* preferences are fixed. This places *Colias* as a rare example in the literature of a polymorphic system without learning that is being maintained by countervailing selection. More work on other species in the Coliadinae could yield valuable inferences about widespread these features are and how countervailing selection maintains polymorphisms across a wide variety environments.

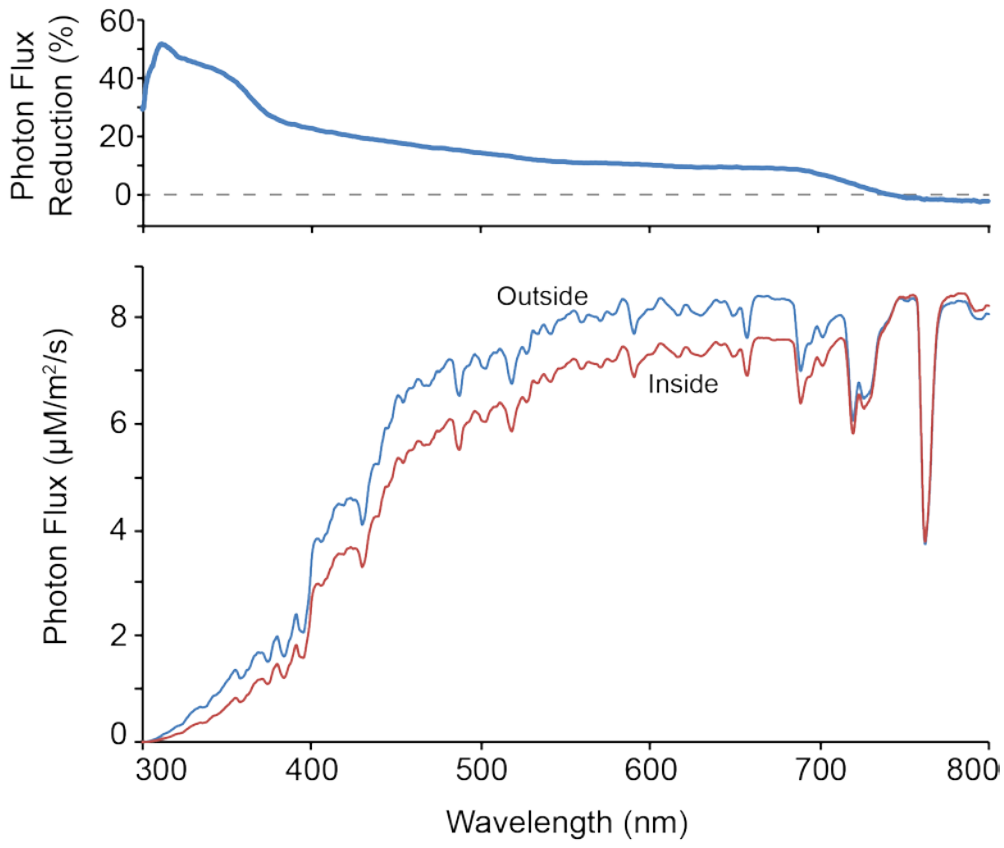
APPENDIX A

SUPPLEMENTARY FIGURES



Supplemental Figure 1. Chromatic and luminance contrasts between *C. philodice* 'alba' females and other butterfly groups.

Comparisons between *C. philodice* 'alba' females and all other groups for forewing (FW) chromatic contrast (A), hindwing (HW) chromatic contrast (B), forewing luminance contrast (C), and hindwing luminance contrast (D). Error bars represent 95% confidence intervals. For symbols where no error bar is visible, error bars are smaller than the size of the symbol itself. For luminance contrast, a positive mean indicates that the group on the X-axis is brighter (has higher luminance) than the group coded by the symbol key.



Supplemental Figure 2. Light transmittance through experimental enclosure fabric.

The photon flux inside and outside of experimental enclosures measuring the light transmittance through the fabric used to construct the enclosures.

APPENDIX B

SUPPLEMENTARY TABLES

Supplemental Table 1. **Visual contrasts between *C. philodice* 'alba' females and other butterfly groups**

Chromatic (ΔS^i) and luminance (L) contrasts (means ± 95% confidence interval) between *C. philodice* 'alba' females and all other groups. For chromatic contrast, values above 2.3 are potentially discriminable under optimal circumstances, and for luminance contrast, values that do not overlap with 0 are potentially discriminable.

Species		
	3 ± 0.03	96.45 ± 6.6

Supplemental Table 2. Visual contrasts between phenotype-altered females and unaltered females

Average color-space contrasts between ten phenotype-altered females and the average spectrum of eight unaltered female reflectance spectra. Chromatic contrast (ΔS_i) and achromatic (luminance, ΔL_{ALL}) contrast are displayed in standard deviations of photoreceptor noise.

Phenotype-altered female	Unaltered female being contrasted	Forewing				Hindwing			
		ΔS_i		ΔL_{ALL}		ΔS_i		ΔL_{ALL}	
		Weber fraction		Weber fraction		Weber fraction		Weber fraction	
		0.01	**0.05**	**0.01**	**0.05**	**0.01**	**0.05**	**0.01**	**0.05**
'alba' -> 'alba'	'alba'	68.7	13.74	0.19	0.19	34.8	6.96	-0.08	-0.08
	non-'alba'	138.7	27.7	-0.03	-0.03	87	17.4	-0.31	-0.31
'alba -> non-'alba'	'alba'	107.5	21.5	0.34	0.34	89.26	17.85	-0.01	-0.01
	non-'alba'	30.97	6.19	0.13	0.13	42.55	8.51	-0.24	-0.24
non-'alba' -> non-'alba'	'alba'	112.7	22.54	0.38	0.38	84.62	16.93	0.1	0.1
	non-'alba'	25.1	5.01	0.17	0.17	38.3	7.67	-0.13	-0.13
non-'alba -> alba'	'alba'	69.4	13.89	0.16	0.16	34.8	6.95	0	0
	non-'alba'	135.7	27.1	-0.06	-0.06	84.3	16.9	-0.23	-0.23

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